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Phylogenetic Variation of CRISPR and Whole genome sequencing of *Salmonella enterica* isolates from clinical samples in Babylon Province

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يَرۡفَعِ ٱللَّهُ ٱلَّذِينَ ءَامَنُواْ مِنكُمۡ وَٱلَّذِينَ أُوتُواْ ٱلۡعِلۡمَ
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Dedication

To my father, *who has always been my role model and greatest mentor and filled me with an overwhelming sense of pride.*

To my mother, *who has overwhelmed me with her love and kindness, the main reason behind my success.*

To my wife, *my dear companion and the candle that lights my way, who has encouraged me to innovate and excel.*

To my kids, *who are the endless love and the secret behind my existence.*

To my sisters and brothers, *who shares me my hopes and supported my efforts.*

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Summary:

A total of 200 clinical stool and blood specimens were collected from patients in the stages of all ages and both sexes suffering from watery diarrhea or with (mucus , pus , little blood or no) and blood specimens obtained from patients with typhoid fever were admitted in three hospitals of Babylon Governorate: the Imam Al-Sadiq Teaching Hospital, Al-Hillah Surgical Teaching Hospital and Babel Teaching Hospital for Women and Children during a period of four months (from February 2022 to May 2022). Then the specimens were cultured in selective media and identified by using bacteriological and biochemical tests (VITEK 2 Compact system) and using specific primer (*SE1472298-2* for *Salmonella enteritidis* , *STM4497* for *Salmonella typhimurium* and the target gene O antigen synthesis *tyv* for *Salmonella typhi*).

Out of 200 specimens , only 34(17%) isolates of *S. enterica* were detected by culture and vitek2 compact system . However , by using specific primers , the result showed only 25 (73.53%) isolates gave positive results for specific primers which included 13(52)% of *S. enteritidis* , 6(24)% of *S. typhimurium* and 6(24)% of *S. typhi* .

Antibiotic susceptibility test was done by vitek 2 compact (card type AST GN76) to detect the microbial sensitive and resistance of *Salmonella enterica* isolates. The isolates used in this study were sensitive for antibiotics. They were highly sensitive for Trimethoprim/Sulfamethoxazole , Imipenem and Tigecycline , at rate (100%) , followed by Piperacillin/Tazobactam , Cefepime and Ertapenem at a rate of (96% , 92 and 92) respectively ; Ceftazidime , Ceftriaxone at a rate of (82%). However , the isolates show moderate sensitive at rate of (64% , 64% and 52%) to Ampicillin , Ciprofloxacin and Nitrofurantoin respectively . Also , the result revealed that some isolates are resistant at a rate of (88%) to Cefazolin , Cefoxitin and Levofloxacin . On the other hand , the isolates were resistant to Amikacin and Gentamicin at rate (92%) . This interpret that these drugs have a good activity against *S. enterica* , because most of them were sensitive to these drugs .

The study implicated the detection of genetic diversity among bacterial isolates which was performed by using CRISPR and were assessed for their potential in 18 *S. enterica* isolates .

A total 18 of *S enterica* serotypes isolates from different clinical specimens (stool & blood) were subjected for detection CRISPR I & II . the result was shown that 18(100%) & 13(72%) isolates were PCR – positive for CRISPR I & II respectively . Direct repeat (DR) of CRISPR I & II isolates showing identical length 29 bp . The polymorphism of DR of CRISPR-I by phylogenetic tree construction indicated that all 18 patterns can divided into six lineage (A-F) , whereas among 13 diverse patterns of CRISPR-II can classified into four lineage (A-D). The genetic similarity value of *S enterica* serotype according to repeat sequence for CRISPR-I and CRISPR-II was also done , the results was shown that in CRISPR-I there is maximum similarity between serotype such as SE11 & STM3 at (100%) and minimum similarity STM4 & STY29 at (40%), while in CRISPR-II , the maximum similarity between serotype SE11 & SE29 at (100%) and the minimum similarity matrix between serotype SE7 & STM4 at (19%).

On the other hand , the CRISPR assay found in *S. enterica* varied in length and spacer content was also studied and the result was shown the number of spacer for CRISPR-I was between 5-13 spacer and CRISPR-II was between 1-12 spacer . However , the alignment comparison of CRISPR-I and CRISPR-II spacer sequence was performed in *S. enterica* .

The phylogenetic tree constructed from the sequence alignment of CRISPR-I spacer show that each serotype was divided in group. In contrast to CRISPR-I, the phylogenetic tree constructed from sequence alignment of the CRISPR-II spacer was unable to discriminate between *S enterica* serovars . Also the genetic similarity value of *Salmonella enterica* (*S typhi* & *S typhimurium*) according to spacer sequence for CRISPR-I and CRISPR-II was also studied , the result was shown that in CRISPR-I there is a maximum similarity between STM26 and STM2 at (100%) and minimum similarity between SE6 and STY29 at (11%) , while in CRISPR-II the maximum similarity between serotypes STM4 & SE7, STM4 &SE8 , STY29 &SE11 at (100%) and the minimum similarity between serotypes STM26 & SE6 at (10%).

Current study also showed that in silico PCR-RFLP of CRISPR was done , the result was shown that the nucleotide sequence of the CRISPR-I and CRISPR-II of S. enterica serovar revealed high diversity in number and nucleotide of spacer and direct repeat . The fragment ranged from 2-330 bp in CRISPR-I , while the fragment ranged from 3-375 bp in CRISPR-II.

At a genomic level, this study tried to discriminate between closely related strains, *Salmonella enterica* subsp. enterica serovar Typhimurium (STY9) and *Salmonella enterica* subsp. enterica serovar Typhi (SalT33) isolates , by using whole genome sequencing . The results of this study noted the chromosomes of *S. enterica* serovars (STY9 and SalT33) are very slightly differences , where the genomic size of STY9 (4687295 bp , 52.2 % GC) was larger than SalT33 (4679911 bp , 52.12% GC) genome . After comparative genome analysis , all the studied genomes of *S. enterica* (STY9 and SalT33) showed different patterns of evolutionary events (genomic rearrangements and segmented gain or loss) with each other or with the reference genome .

In addition , the sequence similarity of *S. enterica* serovars was studied in comparison with the reference genome , the result of this study revealed that the sequence similarity in the SalT33 strain was higher than the STY9 strain , where STY9 have more gaps than SalT33 Following annotation of these genomes , this study reported the presence of (4,701CDS , 38 tRNA , 3 rRNA and 521 Hypothetical proteins) in STY9 genome , (4,855 CDS , 68 tRNA , 4 rRNA and 530 Hypothetical proteins) in SalT33 genome .

All subsystem proteins and Specialty proteins (antibiotic resistance gene , drug targets , transporters and virulence factors) were annotated in this study , where slightly difference between STY9 and SalT33. Subsequently , variant calling for *S. enterica* serovars (STY9 and SalT33) were identified in comparison with the reference genome . According variants count of studied genomes showed the existence of (25608) variants included 99.01% (25355) SNPs , 0.53%(134) insertion and 0.46%(119) deletion in STY9 genome whie , (680) variants included 93.97%(639)SNPs , 2.95%(20) insertion and 3.08% (21) deletion in SalT33 genome.

Correspondingly , the base change count on every SNPs was calculated to identify the type of sequence variation. All studied genomes showed similarity variation patterns , where the most common patterns of base substitution were C ↔ T and G ↔ A substitutions. These patterns represent Transition substitution rather than Transversion substitution in all studied genomes after SNPs. In conclusion , the results of this study provide a comprehensive frame work for under standing the whole genome of *S. enterica* .

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List of Abbreviations

Abbreviation	Meaning
AFLP	Amplified fragment length polymorphism
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Tests
ATR	Acid Tolerance response
a_w	Water activity
BGA	Brilliant green agar
BHI	Brain heart infusion
bp	Base pair
BRIG	BLAST Ring Image Generator
cAMP	Cyclic adenosine monophosphate

Cas	CRISPR associated
CDC	Centers for Disease Control and Prevention
CDS	Coding sequences
cGMP	Cyclic guanosine monophosphate
CGS	Candidate gene sequencing
CLSPT	CRISPR locus spacer pair typing
CRISPOL	CRISPR Polymorphism
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
cse	Chromosome segregation
D.W.	Distilled water
DNA	Deoxyribonucleic acid
DR	Direct repeat
EC	Enzyme commission
EMB	Eosin methylene blue
FAO	Food and Agriculture Organization
FQ	Fluoro quinolones
GC	Guanine cytosine
GO	Gene ontology
HGT	Horizontal gene Transfer
ICMSF	International Commission on Microbiological Specifications for Foods
IL	Interleukin
KEGG	Kyoto Encyclopedia of gene and genomes
KIA	*Kligler Iron Agar*
LCB	Locally collinear blocks
LPF	Long polar flagella
M	Microfold cell
Mac	*MacConkey agar*
MDR	Multi drug Resistance
Mes	Methylation sequencing
MGEs	Mobile Genetic Elements

MIC	Minimum Inhibitory Concentration
MLN	Mesenteric lymph node
MLST	Multi locus sequence typing
MRVP	Methyl Red Voges-Proskauer
MTBC	*Mycobacterium tuberculosis* complex
MVLST	Multi-virulence locus sequence typing
NGS	Next generation sequencing
NTS	Non-typhoidal *Salmonella*
PCR	Polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PHE	Public Health England
PMNs	Polymorphonuclear leukocytes
PMQR	plasmid-mediated quinolone resistance
PPi	Pyrophosphate
RFLP	Restriction fragment length polymorphism
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rrn	Ribosomal RNA
rRNA	Ribosomal RNA
S.S Agar	*Salmonella-Shigella* Agar
SCV	*Salmonella* contain vacuole
SE	*Salmonella enteritidis*
SNP	Single nucleotide polymorphism
SoLiD	Sequencing by oligonucleotide ligation and detection
SPA	Salmonella paratyphi A
SPI	*Salmonella* Pathogenicity Island
STM	*Salmonella typhimurium*
STY	Salmonella typhi
T3SS	Type III Secretion System
Tafi	Thin aggregative fimbriae
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TMP-SMX	Trimethoprim-Sulfamethoxazole

TNF	Tumor necrosis factor
tRNA	Transfer RNA
TS	Typhoidal *Salmonella*
tyv	tyvelose epimerase
UV	Ultra violet
WES	Whole exome sequencing
WGS	Whole Genome Seguencing
WHO	World Health Organization
WTS	Whole transcriptome sequencing
XLD	Xylose lysine deoxycholate

Introduction

&

Literature Review

1.1. Introduction:

Salmonella enterica are a group of Gram negative, motile, facultative anaerobic rod-shaped bacteria belonging to the *Enterobacteriaceae* family (Giner-Lamia *et al*., 2019).

Salmonella enterica infections remain one of the most common foodborne diseases, which constitutes a global public health concern (Jiang *et al*., 2021). As early as the 1950s, *Salmonella* was highlighted as an important zoonotic pathogen with economic implications by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations (Akinola *et al*., 2019). The genus *Salmonella* is divided into two species, *i.e., S. enterica* and *S. bongori*. *S. enterica* is also subdivided into six subspecies and comprises more than 2600 serovars (Jajere, 2019). Pathogenesis of *Salmonella* is mediated by various genes that promote host cell invasion, intracellular survival, and colonization (Mthembu *et al*., 2019).

Non typhoidal *Salmonella* (NTS) serovars that typically cause gastroenteritis can also cause invasive systemic infections (Seribelli *et al*., 2020). Nowadays, invasive NTS infections, which cause an estimated 535,000 cases and > 77,000 deaths annually, are increasingly becoming an important public health threat in low- and middleincome countries, and have gained greater recognition as an important disease in children younger than 5 years (Kariuki & Owusu-Dabo, 2020).

Typhoid fever and other invasive salmonellosis continue to cause an estimated 14.8 million cases and > 200,000 deaths annually, largely affecting children in low- and middleincome countries (Duff *et al*., 2020). Enteric fever, the collective term for typhoid and paratyphoid fevers, describes a systemic infection caused by *Salmonella enterica* serovar Typhi or Paratyphi A, B, or C (Carey *et al*., 2020).

The pathogenesis of these organisms depends on multiple virulence and antimicrobial resistance (AMR) genes (Ngoi *et al*., 2015), mostly associated with *Salmonella* pathogenicity islands (SPIs), prophages, and plasmids. Some virulence factors seem to be conserved among *Salmonella*, while others may be serotype-specific (Hassena *et al*., 2021). Most cases of *Salmonella*-caused diseases are mild, but sometimes they can be life threatening and, therefore, require appropriate antibiotic therapy. Antimicrobial resistance is a public health problem worldwide, and *Salmonella* is among the microorganisms with a number of resistant strains found in the food chain. Ciprofloxacin is a common first-line antimicrobial drug for the treatment of salmonellosis, but since fluoroquinolones are not used to treat children, beta-lactams (ampicillin or third-generation cephalosporins) are equally important (Medalla *et al*., 2017). MDR *S. enterica* serotypes are associated with higher morbidity outcomes compared to drug-susceptible strains (Hindermann *et al*., 2017).

The CRISPR-Cas system is an acquired immune system that protects bacteria from Mobile genetic elements (MGEs), including viruses, plasmids, and transposons (Koonin & Makarova, 2019). The genome architecture of a CRISPR-Cas locus typically has three parts: sequence of CRISPR arrays, a cas gene locus, and AT-rich leader region (Zhang & Ye, 2017). The CRISPR arrays consist of direct repeat sequences of 21-48 base pairs (bp) separated by 26-72 bp long spacer sequences. The spacers are 4-10 highly conserved short nucleic acid sequences obtained from previous encounters with MGEs (Wang *et al*., 2020). The mechanism of action of the CRISPR-Cas system is generally divided into three stages: acquisition of new spacers (the adaptation stage), crRNA biogenesis (the CRISPR transcripts), and interference against foreign invaders directed by crRNAs (Mosterd *et al*., 2021).

Overall, the CRISPR-Cas system is divided into three types: types I, II, and III (Shabbir *et al*., 2019). *S*. enteritidis have a type I-E CRISPR system and consists of a cas operon and two CRISPR arrays, CRISPR1 and CRISPR2, separated by 16 bp (Kushwaha *et al*., 2020). The cas operon is located next to the CRISPR1 array (Koonin & Makarova, 2019) and consists of a cluster of cas3, cas2, cas1, cas6e, cas7, cse2, and cse1 and cas5 genes (Shariat *et al*., 2015) .Apart from defending bacteria against invaders, the CRISPR-Cas system has been suggested to increase bacterial virulence, but its role in antibiotic resistance is still under debate (Shabbir *et al*., 2019).

During the last two decades, whole-genome sequencing (WGS) has become the affordable tool that has the capacity to revolutionize different domains including genetics, microbiology, epidemiology and public health surveillance. The evolution of the current WGS technologies allowed to rapidly increase the realization of bacterial genome sequencing projects (Hu *et al*., 2021) . Since the entire genome is readily available for analysis, WGS has the capacity to replace traditional methods for characterizations such as serotyping, virulotyping and antimicrobial resistance which can easily be predicted from the genome. This improves the capacity of surveillance systems to quickly provide information on the probable source, to identify the path of disease transmission within a population and to detect the virulence factors of the pathogen. Therefore, WGS-based analyses are becoming the primary subtyping tool of choice for pathogens particularly *Salmonella* species (Deng *et al*., 2015).

Alternative methods such as clustered regularly interspaced short palindromic repeats (CRISPR), and whole genome sequencing (WGS), have been proposed to replace conventional *Salmonella* serotyping methods (Tang *et al*., 2019). CRISPR compares spacers of CRISPR loci and cluster strains based on their spacer content similarity (Fabre *et al*., 2012). Lastly, WGS differentiates virtually all strains by detecting variation across the complete bacterial genome (Sandora *et al*., 2020). Although each method has advantages and disadvantages regarding speed, cost, strength, and sensitivity (Zou *et al*., 2013), these techniques can identify geographically dispersed outbreaks at an earlier stage (Mughini-Gras *et al*., 2014).

Aim of study:-

The study is aimed to elucidate of phylogenetic diversity and whole genome sequencing of *S. enterica* strains isolated from clinical specimens.

Objectives:-

1- Isolation and detection of *Salmonella enterica* from blood &stool specimens by traditional methods and vitek 2 compact system.

2- Molecular diagnosis of *Salmonella enterica* by using specific primer.

3- Studying of antibiotic susceptibility test to *Salmonella enterica* by using vitek 2 compact technique.

4- Identification the phylogenetic diversity by CRISPR arrays and applying the *In silico* PCR- RFLP technique as a tracking tool for determining the relationship of *Salmonella enterica* serotypes isolated.

5- Whole Genome Sequencing by NGS for *Salmonella enterica* strains

6- Comparative genome analysis by using modern bioinformatics tools.

1.2 Literature Review

1.2.1 General Characteristics of *Salmonella*

Salmonella represents a large genus of global public health significance and is the leading cause of foodborne illnesses responsible for thousands of deaths worldwide (Odoch *et al*., 2017) . *Salmonella* is Gram-negative, rod-shaped bacteria, and facultative anaerobes belonging to the family Enterobacteriaceae. The genus *Salmonella* belongs to two broad species namely *Salmonella enterica* and *Salmonella bongori*. So far, more than 2600 serovars belonging to *S. enterica* have been described worldwide, and many of these serovars are capable of causing illnesses in both humans and animals (Mezal *et al*., 2014). *Salmonella* species are non spore forming , predominantly motile enterobacteria with cell diameters between about 0.7 and 1.5 µm, lengths from 2 to 5 µm, and peritrichous flagella (all around the cell body) (Fàbrega & Vila, 2013)

They are chemotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. They are also facultative aerobes, capable of generating ATP with oxygen ("aerobically") when it is available, or when oxygen is not available, using other electron acceptors or fermentation ("anaerobically")(Fàbrega and Vila, 2013).

Muktaruzzaman *et al*. (2010) were mentioned that *Salmonella enterica* organisms showed different cultural characteristics in different media. These were turbidity in Tetra Thionate broth , pink white color colonies in Brilliant Green agar , gray white colony in Nutrient agar , slightly grayish color colonies in Salmonella Shigella agar , black color colony in Tripple Suger Iron agar, pale color colonies in MaConkey's agar, well defined glistening colonies in Blood agar and pinkish colonies in eosin methylene blue agar.

These organisms are oxidase negative, catalase positive, indole and voges-proskauer negative, methyl red and citrate utilization positive (Sedeik *et al.*, 2019). They are able to reduce nitrates to nitrite, able to ferment glucose and mannose, with H_2S gas production; non fermenting lactose and sucrose on triple-sugar iron agar and usually able to use citrate as the carbon source solitary (Meneses, 2010).

Salmonella are resistant to certain chemicals such as brilliant green, sodium tetrathionate and sodium deoxycholate that cause inhibition to other enteric bacteria (Robinson, 2014).

Salmonella spp. has relatively simple nutritional requirements and can survive for long periods of time in foods and other substrates; the growth and survival of *Salmonella* spp. is influenced by a number of factors: temperature, pH, water activity and the presence of preservatives (Podolak *et al.*, 2017).

The temperature range for growth of *Salmonella* spp. is 5.2 – 46.2 C^{o}, and the optimum temperature is 35 – 43C^{o} (ICMSF, 1996). Although freezing can be detrimental to *Salmonella* spp. survival, it does not guarantee cause destroy of the organisms. Initially there is rapid decrease in the number of viable organisms at temperatures close to the freezing point as a result of the freezing damage. Strawn & Danyluk (2010) showed that *Salmonella* was able to survive on frozen mangoes and papayas stored at -20 C^{o} for at least 180 days .

Salmonella spp. are grow in a broad pH range of 3.8 – 9.5, the optimum pH range is 7 – 7.5 and the minimum pH at which *Salmonella* spp. can grow is depend on the temperature, presence of salt and nitrite and the type of acid present; volatile fatty acids are more bactericidal than organic acids such as lactic, citric and acetic acid; outside of the pH range for growth; the cells may become inactivated, although this is not immediate and the cells have been shown to survive for long periods in acidic products(Hocking, 2016).

Outcomes of exposure to *Salmonella enterica* non-typhoidal. can range from having no effect, to colonization of the gastrointestinal tract without symptoms of illness (asymptomatic infection), or colonization with the typical symptoms of acute gastroenteritis. Gastroenteritis symptoms are generally mild and may include abdominal cramps, nausea, diarrhoea, mild fever, vomiting, dehydration, headache and/or prostration. The incubation period is 8–72 hours (usually 24–48 hours) and symptoms last for 2–7 days (Darby & Sheorey, 2008).

Enteric fever (typhoid and paratyphoid fever) is caused by *Salmonella enterica* serovar Typhi *(S.* Typhi*)* and *Salmonella enterica* serovar Paratyphi *(S.* Paratyphi). *S.* Paratyphi A and B (and, uncommonly, *S.* Paratyphi C) cause a disease that is clinically indistinguishable from typhoid fever, particularly in parts of Asia. Typhoid fever is an acute, life-threatening, febrile illness. Without treatment, the case fatality rate of typhoid fever is 10–30%, dropping to 1–4% with appropriate therapy. Young children are at greatest risk. Common symptoms include sustained fever, chills and abdominal pain (Organization, 2018).

1.2.2 Taxonomy, Classification and Nomenclature of the *Salmonella*

There are several problems and a lot of proposals in the nomenclature of the genus *Salmonella* According to Bergey's Manual (Palleroni & In, 1984) . Brenner *et al.*, (2000) were reported the nomenclature used at Centers for Disease Control and Prevention for members of the genus *Salmonella* the taxonomy of *Salmonella* is according Jawetz *et al* ., (2013) and it is include:-

Domain : Eubacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Salmonella*
Species: *Salmonella enterica*

The genus *Salmonella* can be classified into only two species according to DNA relativeness *S. bongori*, which inhabits cold blooded animals and *S. enterica*, which is able to inhabit both; cold and warm blooded hosts (Monte *et al*., 2021) .

The species *S. enterica* is divided into the six subspecies *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *Indica* (VI). Formerly, *S. bongori* was the subspecies V, but later considered as a separate species (Chand *et al*., 2020). Strains belonging to *S. enterica* subsp. *enterica* cause approximately 99% of *Salmonella* infections in humans and warm-blooded animals (Yousif & Harab, 2011).

The nomenclature of *Salmonella* is now based on the name of serotypes belonging to subspecies. For example, *Salmonella enterica* subsp. *enterica* serotype *Typhimurium* is shortened to *Salmonella Typhimurium* (Brenner *et al*., 2000).

Salmonella is the a large genus, and the serotyping of it is widely used to classify isolates into serogroups based on their surface antigenic structure variability. Serotyping is based on the immunological classification of the lipopolysaccharides moieties (O antigen), the flagellar protein (H antigen)and the capsular polysaccharide (Vi antigen). The Kauffman-White scheme is generally used for the classification of *Salmonella* serotypes , it is recognizes 64 O serogroups and 114 H antigens resulting in 2668 characterized serotypes (Akbarmehr, 2012).

The O-antigen that designated A, B, C, and so on. For further classification, the flagellar H antigens have been employed. H antigens are two types: specific phase or phase 1, and group phase or phase 2. Phase 1 antigens are shared with only a few other species or varieties of *Salmonella*; phase 2 may be more widely distributed among several species (Strockbine *et al*., 2015). Serotypes are identified by antigenic formulae, taking the general form O antigen, phase 1 and phase 2 antigens, in which O antigens are designated by numerals, the phase 1 antigens by small letters, and phase 2 antigens by arabic numerals. Thus, the complete antigenic analysis of *S. enterica* is as follows: 6, 7, c, 1, 5, where 6 and 7 refer to O antigens, c refer to phase 1 flagellar antigens, and 1 and 5 refer to phase 2 flagellar antigens. *Salmonella* subgroups of this type are referred to as serovars.

Salmonella serotypes are normally divided into two groups these are based on host range; host adapted and host restricted (ubiquitous); the first group of host adapted serotypes, such as *Salmonella* ser. Typhi, *Salmonella* ser. Paratypi A, *Salmonella* ser. *Paratyphi* C they are only infects humans; the second group of host adapted serotypes, such as *Salmonella* ser. Dublin and *Salmonella* ser. *Gallinarum* can cause the disease in more than one host species. Host restricted serotypes, for example *Salmonella* ser. *Typhimurium* and *Salmonella* ser. *Enteritidis*, which are pathogenic for both humans and animals (Uzzau *et al*., 2000). The subspecies name does not need to be indicated, as only serovars of subspecies *enterica* has a name. Therefore one writes e.g. *Salmonella Typhimurium* (Crum-Cianflone, 2008).

1.2.3 Pathogenesis of *Salmonella*

Salmonella enterica is a versatile gastrointestinal pathogen with the ability to cause diseases ranging from acute, usually self-limiting gastroenteritis due to infections by non-typhoidal *Salmonella* (NTS) to severe systemic infections caused by typhoidal *Salmonella* (TS) serovars. Infections by *S. enterica* serovars such as Typhi (STY) and Paratyphi A (SPA) represent a continuing threat to human health (Saleh *et al*., 2019).

After the ingestion of contaminated food or water, *Salmonella* colonizes the distal ileum and proximal colon (Lönnermark *et al*., 2015 ; Hocking, 2016) . The infective dose for salmonellosis that is capable of establishing infection in the mucosa of the small intestine ranges from 10^5 to 10^6 cells (Xu *et al*., 2010). *Salmonella* uses its flagella as a mode of movement as well as chemotaxis to target cells, the enterocytes. In humans, Salmonella cells use type I fimbriae, including long polar fimbriae (Lpf) and thin aggregative fimbriae (Tafi), to adhere to enterocytes. Type IV pili are used by *S. Typhi* to attach to host cells (Wagner & Hensel, 2011).

Salmonella pathogenesis is mainly attributed to the function of two type three secretion systems (T3SS-1 and T3SS-2) encoded on Salmonella pathogenicity Islands 1 and 2 (SPI-1 and SPI-2) (Marcus *et al*., 2000). The T3SSs are needle-like complexes used by bacteria to deliver effectors directly into the cytoplasm of host cells. SPI-1 present in all serovars of *S. bongori* and *S. enterica*, encode the T3SS-1 genes important for *Salmonella* invasion of intestinal epithelial cells. While SPI-2 present exclusively in *S. enterica* serovars, encode T3SS-2 genes important for intracellular survival inside host cells (Fàbrega & Vila, 2013).

Salmonella infections begins with the ingestion of the organism in contaminated food or water. To survive the acidic pH of the stomach, *Salmonella* induces the acid tolerance response (ATR)using about fifty heat shock proteins, which promotes survival, by inducing a pH homeostasis that maintains the bacteria internal pH at values higher that the extracellular environment, (Spector & Kenyon, 2012).

After surviving the acidic condition of the stomach, *Salmonella* travels to the small intestine where it uses flagella motility and chemotaxis to identify cells (at the apical surface of intestinal epithelia) that are permissive for invasion (Pham & McSorley, 2015). *Salmonella* preferentially penetrates the intestinal epithelial through microfold (M) cells. However, it can also penetrate via dendritic cells, intestinal epithelia cells and through breaches in the epithelia lining (Crump *et al.*, 2004). *Salmonella* initiates contact with permissive cells using adhesive proteins such as fimbriae. This contact is further stabilized by SPI-1 T3SS effectors which also induce the formation of membrane ruffles at the site of attachment and the internalization of *Salmonella* through micropinocytosis (Fàbrega & Vila, 2013). Once engulfed Salmonella is internalized into large vesicles called *Salmonella*-containing vacuoles (SCV). SCV is the only intracellular compartment in which *Salmonella* survives and replicate, in eukaryotic cells (Fàbrega & Vila, 2013). Inside SCV, effectors secreted by SPI-2 T3SS prevents the fusion of SCV with lysosome, promote SCV maturation, and manipulate the host cell environment to promote Salmonella survival inside SCV. After traversing the epithelia cells, *Salmonella* are taken up by phagocytic cells at the sub mucosa (Crump *et al.*, 2004).

Once *Salmonella* has adhered to the host cells on the apical side of M cells or enterocytes, it uses Salmonella pathogenicity islands (SPIs) – encoded type III secretion systems (T3SSs) – to be phagocytized into the receptive macrophages (Wagner & Hensel, 2011). *Salmonella* cells can then be exocytosed into the interstitial spaces of the lamina propria, where they are randomly picked by macrophages, dendritic cells and polymorphonuclear cells and distributed to the host efferent lymph in the mesenteric lymph nodes before being transported to the spleen and liver via the bloodstream (Wiedemann *et al.*, 2012). The attachment of Salmonella to the receptive epithelial cells and internalization into lamina propria causes inflammatory responses, including the release of pro-inflammatory cytokines. Pro-inflammatory cytokines cause acute inflammatory responses which lead to diarrhoea, ulceration and the destruction of the mucosa cells (Gut *et al.*, 2018). Apart from the invasiveness of Salmonella cells, enterotoxin and cytotoxin have been identified across all of the Salmonella sub-species. These toxins are reported to be similar to cholera toxins. Some of them have been found to be either heat-labile or heat-stable, and they have been reported to be associated with diarrhoea (Song *et al.*, 2013). Enterotoxin was reported to induce the accumulation of fluid in the ligated murine ileal loop and was also found to have cytotoxic activity (Gut *et al.*, 2018). Cytotoxin inhibits protein synthesis, and it has been reported that it is responsible for intestinal mucosal surface damage, as well as enteric symptoms and inflammatory diarrhoea (Hocking, 2016).

Unlike TS serovars which disseminate to systemic site through phagocytic cells, NTS infection is restricted to the gut (Johnson *et al*., 2018). During invasion and intracellular survival, effectors secreted by NTS induce an inflammatory response which leads to the recruitment of polymorphonuclear lymphocytes (PMNs) and phagocytic cells, that are effective in eliminating NTS using mechanisms such as: activating infected macrophages, production of reactive oxygen and nitrogen species (ROS and RNS), and stimulating epithelia cells to release antimicrobial peptides (Crump *et al*., 2004). TS are distinct from NTS serovars in that they transverse the intestinal epithelia without inducing inflammatoion. TS taken up by phagocytic cells at the submucosa are carried to other sites in the body such as the liver, spleen, MLN, and gall bladder, where they can establish chronic infection (Johnson *et al*., 2018).

The severity of *Salmonella* infections in humans varies depending on the serotype involved and the health status of the human host. Children below the age of 5 years, elderly people and patients with immunosuppression are more susceptible to *Salmonella* infection than healthy individuals. Almost all strains of *Salmonella* are pathogenic as they have the ability to invade, replicate and survive in human host cells, resulting in potentially fatal disease (Eng *et al*., 2015).

1.2.3.1 Mechanism of diarrhea

Salmonella is a common cause of bacterial diarrhea in children, and has an especially high incidence in infancy. It is one of the major causes of foodborne bacterial enterocolitis in many countries (EMRO *and* WHO, 2016). A study done by Berk (2008) who found when *Salmonella* has attached the intestines, has an effect on the intestinal cells which lead to diarrhea; voluminous diarrhea is the classic feature of clinical

Salmonellosis in most vertebrate species; this is as a result of losses intestinal fluid by two mechanisms: active fluid loss through secretory hyper-stimulation and passive fluid loss by inflammation mediated malabsorption (Chapman, 2006).

The development of diarrhea is mediate by various toxins by *Salmonella* these toxins include: exotoxin, cytotoxin, enterotoxin and endotoxin; cytotoxin as the name implies, causes damage to the intestinal epithelial cell either directly through chelating of cations in the mucosal cell membrane or indirectly via stimulation of cytokines and inflammation (Chapman, 2006).

In addition *Salmonella* causes intestinal epithelial cell damage of absorptive villous enterocytes; these are lead to reduce the capacity of absorption and result loss of electrolytes and water (Chapman, 2006). Another mediator of inflammation is endotoxin (bacterial LPS), which through its interaction with local macrophages triggers a profound inflammatory effect resulting in the influx of the neutrophils, inflammatory mediators released by infiltrating leukocytes, such as cytokines (interleukin IL-1, IL-6, IL-8 and TNF-α tumour necrosis factor), enzymes and oxygen species provoke local tissue damage and contribute to the breach in mucosal integrity and intestinal malabsorption(McCormick *et al*., 1995; Jones & Blikslager, 2002).

Salmonella enterotoxins has been initiate diarrhea by binding to receptors that stimulate the second messenger systems of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which secondarily activate enterocyte fluid hypersecretion (Jones & Blikslager, 2002) .

On the other hand, *Salmonella* is invasion of the intestinal epithelial cells and has been released cytotoxin that acts to exhibit the proteins production within the epithelial cells that lead to its damage and convert into articles of pus and mucus with the appearance of blood in the stool to turn away from watery diarrhea to bloody diarrhea (Feasey *et al*., 2012).

1.2.4 Clinical Manifestations of *Salmonella*

Infection of humans with *Salmonella* results in three main infectious diseases, namely typhoid fever, paratyphoid fever and NTS. Typhoid and paratyphoid fevers are caused by S. Typhi and *Salmonella* enterica serovar Paratyphi (S. Paratyphi), respectively, and are characterized by gastroenteritis and complications such as septicaemia, immunological symptoms, leukopenia and neurological sympotoms. These typhoidal and paratyphoidal complications account for deaths (Andino & Hanning, 2015 ; Chong *et al*., 2017). On the other hand, *S. Typhimurium, S. Enteritidis* cause NTS infections, which are restricted to gastroenteritis (nausea, vomiting and diarrhoea) or occasional bacteraemia (dissemination of infection in the body), and are usually non-fatal (Andino & Hanning, 2015).

1.2.4.1 Typhoidal *Salmonella*

Typhoid fever or as well called" enteric fever " is a food-borne and a world wide spread disease (Eissa *et al*., 2018) .Typhoid fever is most predominant in the Asian part of the world especially in the developing countries of Asia, caused by *Salmonella enterica* subspecies *enterica* serovars *Typhi* , *Paratyphi A* and *Paratyphi B* cause enteric fever (Kasumba *et al*., 2021).

It is an orally transmitted communicable disease caused by consuming contaminated food and impure water (Nusrat *et al.,* 2018). It passes through the stomach and invades the gut epithelium, possibly in the distal ileum (Dougan & Baker, 2014).

The organisms attach themselves to the epithelial cells of the small intestines, penetrate the sub-mucosa, and pass from there into the blood stream via the lymphatic. A transient bacteremia follows and the bacteria seed the reticulo endothelial system (liver, spleen, bone marrow) and the gall bladder and kidneys (Ugboko & De, 2014). The organisms reenter the intestine from the gall-bladder where it involves the Payers patches, inflammation and ulceration. The incubation period is about 5 to 21 days (Kaur & Jain, 2012).

Enteric fever is a life-threatening disease cause by TS serovars: Typhi and Paratyphi. After ingestion TS serovars have an asymptomatic period of 7 to 14 days and once developed symptoms can persist for up to 3 weeks. The predominant symptom is fever due a gradual rise in temperature up to 40°C. Other reported symptoms include, chills, abdominal pain, hepatosplenomegaly, rash (rose spots), nausea, anorexia, diarrhea or constipation, headache, and dry cough (Gal-Mor *et al*., 2014).

Fever is typically mild at first and worsening as disease progresses (Parry *et al*., 2002). In the absence of complications, disease resolves following varied periods of infection although carriage of the bacteria can continue in post-symptomatic patients for months or years and relapse occurs in a minority of patients (Coburn *et al*., 2007).

Its mortality has been significantly reduced by implementation of appropriate antibiotic treatment but the increase in betalactam and fluoroquinolone resistance of *Salmonella* strains is a growing public health problem (Rödel *et al*., 2020).

1.2.4.2 Non-typhoidal *Salmonella*

Salmonella infection or a disease associated with it, *salmonellosis* is most often characterized by enteritis, gastroenteritis is commonly associated with NTS serovars such as *typhimurium* (*S. typhimurium*) and *enteritidis* (*S. enteritidis*). However, host restricted serotypes tend to induce higher levels of bacteremia, while some human restricted serotypes cause a systemic disease that is characterized by mild symptoms (Gal-Mor *et al*., 2014). Children are the most likely group of individuals to present *Salmonellosis*. The rate of diagnosed infections in children <5 years old is higher than the rate diagnosed in all other persons. Other groups of risk, such as elderly and immune compromised individuals are the most likely to present severe forms of the disease (Hurley *et al*., 2014).

Gastroenteritis is predominately caused by NTS serovars: Typhimurium and Enteriditis. Symptoms appear 6 – 12 hours after ingestion and persist for less than 10 days. The most common initial symptoms reported are nausea, vomiting and watery diarrhea. Other symptoms include fever, chills, abdominal pain, and headaches (Gal-Mor *et al*., 2014). NTS infection is usually limited to the gastrointestinal tract, however, about 5% of patients develop secondary bacteremia when bacteria disseminate to other parts of the body (Gal-Mor *et al*., 2014). NTS gastroenteritis is usually self-limiting in immunocompetent individuals, and severe cases can be treated with antibiotic (Onwuezobe *et al*., 2012).

Persons with *Salmonella* infections that develop pain in their joints, irritation of the eyes, and painful urination. Taken together, these symptoms indicate a disease called reactive arthritis, this disease can persist for months or years, and can lead to chronic arthritis, which is extremely difficult to treat, death from salmonellosis can be caused by perforation of the gut and necrosis of Peyer's patches leading to peritonitis or toxic encephalopathy (Parry *et al.*, 2002).

The continuous evolution of *Salmonella* at the genetic and genomic levels contributes to the increased virulence and resistance to multiple antibiotics, leading to a phenotype of multidrug resistance. This resistance is a significant public health concern (Fàbrega & Vila, 2013).

Two major changes in the epidemiology of non-typhoidal *Salmonellosis* have occurred in the last century. These were the emergence of food-borne human infections caused by *Salmonella enterica enteritidis* and by multidrug-resistant strains of *Salmonella enterica typhimurium*. In this century, a concerning situation is the increased resistance that non-typhoidal *Salmonella* (NTS) presents to fluoroquinolones and third-generation cephalosporins clinical isolates showing carbapenem resistance have also being reported (Wang *et al.*, 2013).

However, previous studies indicated that a 3–5 days therapy with ceftriaxone for patients with severe gastroenteritis could lead to a faster recovery (Hurley *et al.*, 2014).

1.2.5 Treatment of *Salmonella enterica*

A wide variety of antimicrobial agents are available today and they are broadly applied to treat different types of human infections. Specifically, antibiotics are powerful drugs used for treatments of pathogenic bacteria (Lei *et al*., 2019). However, their indiscriminate and prolonged use, especially in developing countries, in both human and veterinary medicine, as well as in agriculture have contributed to the development and spread of drug-resistant microorganisms (Huan *et al*., 2020). As the World Health Organization (WHO) has extensively announced, the alarming rise globally in resistance towards conventional antimicrobials represents a potential and serious risk to public health (Luong *et al*., 2020).

Recommendations are to treat most patients with uncomplicated *Salmonella* infection with oral rehydration therapy but not with antimicrobial agents. Antimicrobial therapy should be considered for patients who are severely ill (for example, those with severe diarrhea, high fever, or manifestations of extraintestinal infection) and for people at increased risk of invasive disease (infants, older adults, and the debilitated or immunosuppressed). When antimicrobial therapy is indicated, empiric treatment is usually required until susceptibility data are available. Resistance to antimicrobial agents varies by serotype and geographic region (Brunette, 2017).

In cases of extra-intestinal or focal infection, and similar to bacteraemia, antibiotics are chosen depending on the susceptibility pattern of the strains in cause, considering as well the clinical condition of the patients. Mostly, the therapy includes ampicillin, trimethoprim-sulfamethoxazole, fluoroquinolones or third generation cephalosporins, such as ceftriaxone (Chen *et al*., 2013). With potential risk of causing arthroplasty, fluoroquinolones remain last option in NTS infection for children, when there is no other alternative treatment available (Chen *et al*., 2013; Choi *et al*., 2013).

The greatest challenge of antibiotics therapy is the development of resistance in which mostly is associated with people via drug misuse and gene mutations of microbes (Akond *et al*., 2013). Drug resistant is the ability of a pathogen to resist killing effect of the drugs (Adesiji *et al*., 2014). Testing of individual pathogens against appropriate antimicrobial agent is often necessary since susceptibility of many pathogens such as bacteria to antimicrobial agents cannot be predicted and is done for the sake of preventing emergence of drug resistance to these strains (Yhiler & Bassey, 2015 ; Tsegaye *et al*., 2016).

Many studies have indicated that, there are transfers of genes from environmental bacteria to pathogens via gene mutations which can cause emergence of multiple resistances against antimicrobial agents (Adesiji *et al*., 2014). The dynamics of trend of transmission of zoonotic infections and environmental components which obviously need to be addressed in order to control the escalating spread of these infections. The environmental strains of *Enterobacteriaceae* are usually susceptible to aminoglycosides, Imipenem, Quinolones and third generation Cephalosporins (Phagoo & Neetoo, 2015).

S. enterica is a Gram-negative bacterium with more than 2600 serotypes that, based on their different pathogenic behaviors, can be divided into two groups: typhoidal *Salmonella* and nontyphoidal *Salmonella* (Maka & Popowska, 2016). While typhoidal *Salmonella* is associated with a high number of fatal cases (X. Wang et al., 2019), nontyphoidal *Salmonella* infections in humans are generally self-limiting and do not require antimicrobial treatment (EFSA , 2020).

However, in rare cases, the infection can be more serious, and the use of antimicrobial agents such as fluoroquinolones and third-generation cephalosporins, generally recommended for treating both adults and children, is essential. Salmonella as well as other pathogenic bacteria can exhibit resistance to a wide range of antibiotics, and it has been demonstrated that multidrug-resistant Salmonella infection may have a more serious human health impact compared to infection by less resistant strains (EFSA , 2020).

Enteric fever is a serious bloodstream infection caused by Salmonella enterica serovars Typhi and Paratyphi A and is an important cause of morbidity and mortality (Britto *et al*., 2020).

Early diagnosis and prompt initiation of appropriate antibiotic therapy prevents complications of enteric fever and *Salmonella* bacteremia or sepsis (Riedel *et al*., 2019), But if treatment is delayed or is rendered ineffective by resistance, the complication and case fatality rate increases substantially (Karkey, *et al*., 2018). The mortality rate of enteric fever in the pre antibiotic period was estimated to be between 10 and 30%. The availability of traditional first-line antimicrobials over the past nearly 70 years (Chloramphenicol, Ampicillin, and Trimethoprim-Sulfamethoxazole) has reduced the overall mortality rate to less than 1% (Khair-Allah, *et al*., 2019).

Infections caused by *S. typhi* may involve serious complications and require treatment with antibiotics such as Cefixime, Chloramphenicol, Amoxicillin, Trimethoprim / Sulfamethoxazole (TMP-SMX) , Azithromycin, Aztreonam, Cefotaxime or Ceftriaxone to prevent death (P. Kumar & Kumar, 2017). Third generation Cephalosporins are recommended for treatment of typhoid fever. Cases requiring hospitalization should be treated with Ceftriaxone. In ambulatory patients, Cefixime can be used (P. Kumar & Kumar, 2017).

Azithromycin is effective for the management of uncomplicated typhoid fever and may serve as an alternative oral drug in areas where fluoroquinolone resistance is common. Azithromycin prevents bacteria from growing by interfering with their protein synthesis. It binds to the 50S subunit of the bacterial ribosome, thus inhibiting translation of mRNA. Nucleic acid synthesis is not affected (Crump *et al*., 2015). Azithromycin is a macrolide antibiotic widely used for the empirical treatment of uncomplicated enteric fever, benefitting from once daily oral dosing and good tissue penetration. It has excellent in vitro activity, being concentrated within phagocytic cells and achieving intracellular concentrations of up to 200 times greater than serum. Several randomized controlled trials have demonstrated the efficacy of Azithromycin in adults and children when compared with fluoroquinolones, cephalosporins and chloramphenicol including in the treatment of fluoroquinolone intermediate or resistant strains (Jin *et al*., 2019). Azithromycin is popular because of its oral formulation and single daily dose (Djeghout *et al*., 2018).

1.2.6 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are a special characteristic of a DNA arrays containing conserved short repetitive direct repeat, which are embedded between a short variable sequence called spacer (Rath *et al*., 2015). The nucleotide sequence of spacers is similar to some parts of those derived from invading bacteriophage or plasmid, suggesting that the CRISPR arrays are part of a specific immune system against previous infectious bacteriophage and plasmid (Van der Oost *et al*., 2009 ; Horvath & Barrangou, 2010; Terns & Terns, 2011). It is widely acknowledged that bacteria have a mechanism to uptake and integrate a new spacer DNA from bacteriophage or plasmid into their genome (Barrangou *et al*., 2007; Horvath & Barrangou, 2010). After the acquisition of spacers, the direct repeat and spacers are transcribed as a short RNA called CRISPR-RNA (crRNA). This crRNA coupled together with a CRISPR-associated protein (Cas) subsequently cleave known bacteriophage or plasmids DNA (Marraffini & Sontheimer, 2010;Pougach *et al*., 2012).

Although several studies have been reported that the *S. enterica* contains 2 types of CRISPR arrays in the chromosomes, and application of CRISPR typing and subtyping for improved laboratory surveillance have been reported (Fabre *et al*., 2012 ; Shariat *et al*., 2013 ; Shariat *et al*., 2015).

S. enterica possesses type I–E CRISPR system comprising a *cas* operon and two CRISPR arrays, CRISPR1 and CRISPR2 (Karimi *et al*., 2018), separated by ~ 16 kb (Shariat *et al*., 2015). The *cas* operon present in proximity to the CRISPR1 array (Koonin & Makarova, 2019) contains 8 *cas* genes. Two distinct *cas* gene profiles has been observed with reported incongruence between the *cas* and whole genome phylogeny (Pettengill *et al*., 2014).

A CRISPR locus usually contains two to several hundreds of direct repeat (DR) sequences of 23–50 bp in length, separated by unique spacer sequences of similar length (Pourcel *et al*., 2020). Spacers share complementarity with sequences identified in foreign DNA elements (protospacers) and are acquired from phages, plasmids, and other transferrable elements that previously infected bacteria (Makarova *et al*., 2015; Koonin & Makarova, 2019). To differentiate foreign DNA elements from self-DNA, the Cas proteins follow often at least three-nucleotide long protospacer-adjacent motif (PAM) present on the target sequence (Leenay *et al*., 2016).

The genus Salmonella is known to carry a class-1 type I-E system, closely related to the CRISPR-Cas system in *Escherichia coli* (E. coli)

(Shariat *et al*., 2015). The systems have been reported to carry either one or two CRISPR loci and a cas-gene cluster of cas3, cse1-cse2-cas7-cas5-cas6-cas1-cas2 genes (Medina-Aparicio *et al*., 2017). CRISPR-Cas systems in other bacterial species have been explored extensively for typing purposes (Louwen *et al*., 2014).

Interestingly, it is suggested that the amplification of only the newly incorporated spacers in CRISPR1 and CRISPR2 (CLSPT, CRISPR locus spacer pair typing) is good enough to discriminate *Salmonella* serovars (Li *et al*., 2014). In the case of the etiological agents of typhoid fever and paratyphoid fever, *S*. Typhi and *S*. Paratyphi A, respectively, it is known that CRISPR2 amplification allows the distinction of these two serovars (Fabre *et al*., 2014).

The typing based only on CRISPR arrays allows the characterization of the predominant *Salmonella* populations in dairy farms, in order to prevent dissemination to human hosts (Wehnes *et al*., 2014) . Additionally, the ease of obtaining different length variants of CRISPR1 and CRISPR2 using PCR has allowed the rapid differentiation of *S*. Typhimurium and *Salmonella* Newport outbreak strains, and this simple screening approach is suitable for low-capacity public health laboratories or hospitals (Fabre *et al*., 2012 ; Shariat *et al*., 2013).

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and a set of CRISPR-associated (*cas*) genes are suggested to be acquired by horizontal gene transfer (HGT) event (McDonald *et al*., 2019). The Cas1 and Cas2 proteins are essential for spacer acquisition from invading mobile genetic elements (MGE) (Lamas *et al*., 2018) while all Cas proteins participate in primed adaptation to update the invaders' memory (Krivoy *et al*., 2018). The newly acquired spacers are added at the leader proximal end of the CRISPR array (Lamas *et al*., 2018). Cas proteins work in conjunction with the CRISPR-RNA to carry out the interference step (Gao *et al*., 2017). CRISPR-Cas system has been related to the bacterial virulence potential(Li *et al*., 2016 ; Cui *et al*., 2020) The number of CRISPR array are negatively correlated with pathogenic potential of *Escherichia coli* where, the reduction in CRISPR activity is proposed to promote HGT favouring its evolution (García-Gutiérrez *et al*., 2015).

Conversely, some reports demonstrate a positive correlation between the CRISPR and pathogenicity owing to virulence genes regulation (Li *et al*., 2016 ; Cui *et al*., 2020).

In *S. enterica* subsp. *enterica* serovar Enteritidis, Cas3 modulates biofilm formation and virulence by regulating quorum sensing genes (Cui *et al*., 2020). Further, in *Salmonella* and *E. coli*, 53% of CRISPR protospacers traced to chromosomes (Touchon & Rocha, 2010) suggesting a potential role of the CRISPR-Cas system in endogenous gene regulation (Nguyen *et al*., 2018) and possibly pathogenesis (Cui *et al*., 2020).

Regarding the highly clonal Enteritidis serovar, one of the major causes of food-borne salmonellosis in the United States, several reports show that CRISPR- Multi-virulence-locus sequence typing (MVLST) together with PFGE are highly accurate typing methods to discriminate chicken, egg, environmental and human clinical isolates (Liu *et al*., 2011). This methodology has also made possible the identification of the predominant Enteritidis sequence types present in specific geographical regions (Deng *et al*., 2015) or to determine that the origin of human infections in Tahiti was derived from farm animals (Hello *et al*., 2015).

In other serovars such as Typhimurium, a bacterium able to induce gastroenteritis in humans, it has been demonstrated that the CRISPR-MVLST is a very efficient approach for subtyping Brazilian isolates, since it complements and validates the results obtained by pulsed-field gel electrophoresis (PFGE) (Almeida *et al*., 2017). In addition, a relation between CRISPR sequence types and antibiotic resistance was observed in this *Salmonella* serovar present in animals and human isolates (DiMarzio *et al*., 2013). Furthermore, based on CRISPR polymorphism, the (CRISPOL) CRISPR polymorphism method was developed for Typhimurium, allowing the creation of an open web-accessible database, providing a serotype/spacer dictionary internationally available for strain tracking (Fabre *et al*., 2012).

The CRISPR arrays have diverse aspect for applications in biomedical science. For example, the use of CRISPR arrays as genetic markers in the epidemiological study, especially for outbreak investigations which have been proved to be efficient in several pathogenic bacteria. It was first described in *Mycobacterium tuberculosis* complex (MTBC), which indicated that each different strain of MTCB contains polymorphisms in the CRISPR arrays (Groenen *et al*., 1993;Kamerbeek *et al*., 1997). A comparative study of genotyping for outbreak tracking of *Campylobacter jejuni* by CRISPR sequencing, amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) demonstrated that the power of discrimination of those three techniques was closely related (Schouls *et al*., 2003). The high resolution discrimination by CRISPR genotyping was also shown in *Yersinia pestis* (Pourcel *et al*., 2005).

In the case of *S. enterica*, an enteric pathogen commonly causes of foodborne illness in human, it has tremendous diversity in the subspecies and serovar levels since it comprises of six subspecies and more than 2,500 serovars (Akbarmehr, 2012). Considering this, an efficient laboratory method for serotyping is very important for epidemiological study.

1.2.7 Whole genome sequencing

Since the discovery of DNA structure by Watson and Crick (Watson and Crick, 1953). Several molecular approaches were developed to detect the sequence of DNA bases. The pioneering development of these approaches facilitates evolution of the field of DNA sequencing in applications, capacity and capability (Levy & Myers, 2016). Whole genome sequencing (WGS) represents one of these methods that determine DNA bases at the genome scale. WGS provides the most comprehensive identification of genetic variation for any organism (Ng and Kirknes , 2010). WGS of bacterial pathogens has become more available and affordable as a tool for studying the whole genome of bacteria. Moreover, analysis of the Whole genome of pathogen via WGS might provide exceptional discrimination of highly related lineages of bacteria and could develop outbreak analysis by monitoring the evolution of multi-drug resistant bacterial pathogens (Control, 2016 ; Quainoo *et al.*, 2017) .In addition, sequencing multiple genomes provides important insights about the genetic variation and polymorphisms among their genomes that in turn involved in identification of nonsynonymous or synonymous mutations (Cohen *et al.*, 2004 ; Sjoblom *et al.*, 2006).

Progress in whole genome sequencing (WGS) technology has contributed to the high-throughput sequencing of bacterial genomes at reasonable prices, such that WGS has become an alternative to conventional outbreak typing and identification methods in public health (Kwong *et al.*, 2015). Although WGS offers the possibility of resolving bacterial strains at the specific nucleotide resolution required to classify cases with a common infection source (Dallman *et al.*, 2015), Categorizing isolates into higher taxonomic variants (e.g., those identified by serotyping) is a significant step. Public Health England (PHE) has successfully implemented WGS research for *Salmonella* Typhi, which substitutes the traditional serotyping method of *Salmonella* for regular public health surveillance and gives information on the genetic population distribution of Salmonella species in the world (Ashton *et al*., 2016).

The advent and implementation of WGS drastically revolutionized the techniques used not only to identify individual *Salmonella* strains, but also to detect precise types of serovars (Ricke *et al*., 2018). WGS is an economical typing method for outbreak detection and public health surveillance (Kwong *et al*., 2015). Furthermore, the advantage of WGS to address single nucleotide resolution bacterial strains demands the identification of related cases from a common source of infection and the categorization of the isolates into higher taxonomical levels (e.g., those defined by serotyping) in crucially important cases associated with a common source of infection (Dallman *et al*., 2015).

The potential of WGS for characterizing Salmonella outbreaks and differentiating outbreak and sporadic strains has been explored (Leekitcharoenphon *et al*., 2014; Qi *et al*., 2013) and the importance of correlating WGS and epidemiological data has been emphasized (Leekitcharoenphon *et al*., 2014). With increased understanding of the significance of genomic variability, there is potential to progress to near real-time, discriminatory genotyping of outbreak cases (Qi *et al*., 2013).

In addition, WGS-based identification of virulence, antibiotic resistance, and toxin genes could be more suitable and successful than PCR-based identification methods during outbreak circumstances, where this high resolution identification of pathogenicity genes might lead improved virulence characterization and outbreak control (Quainoo *et al*., 2017).

WGS can also be used to identify the path of disease transmission within a population and provide information on the probable source. It is essential for mutation detection and in understanding of genetics of Salmonella and other microorganisms. It can also evaluate the evolution of strains during an outbreak and detect contextual data on the genetic interrelatedness (Gilchrist *et al*., 2015). A major application for WGS is to identify outbreak clusters and efficiently infer phylogenies from the sequencing reads (Ahrenfeldt *et al*., 2017). WGS is used as an alternative technique for obtaining fast and reliable serotype information (Gymoese *et al*., 2017).

WGS is rapidly replacing current molecular subtyping methods for surveillance and for foodborne outbreak purposes (den Bakker *et al.*, 2011 ; Leekitcharoenphon *et al*., 2014). It enables high-resolution molecular subtyping and provides valuable additional data regarding further characterization of emerging clones based on genetic differences and evolutionary studies (Bale *et al*., 2016 ; Bekal *et al*., 2016). This information is critical during any outbreak response for gathering clonal information in outbreak investigations. Several studies have shown that WGS based typing has an enhanced discriminatory power compared to current molecular typing methods used for *Salmonella* (Köser *et al*., 2012 ; Deng *et al*., 2015). Public health microbiology is currently being transformed by next generation sequencing, which opens the door to more rapid serotype determination using WGS data. SeqSero (www.denglab.info/SeqSero) is a novel web-based tool for determining Salmonella serotypes using whole genome sequencing data (Zhang *et al*., 2015).

Advances in whole genome sequencing (WGS) methodologies have resulted in the ability to perform high throughput sequencing of bacterial genomes at low cost making WGS an economically viable alternative to traditional typing methods for public health surveillance and outbreak detection (Köser *et al*., 2012). Whilst WGS provides the opportunity to resolve bacterial strains to the single nucleotide resolution needed for identifying cases linked to a common source of infection (Dallman *et al*., 2015), grouping isolates into higher taxonomical clones (e.g., those defined by serotyping) is an important step. The decision to adopt WGS as a routine typing method at PHE provided the opportunity to review our approach to typing *Salmonella* and to implement the MLST approach in parallel with WGS (Ashton *et al*., 2016).

The whole genome sequencing (WGS) becomes very useful in public health microbiology around the world. WGS can identify only single nucleotide differences in the genome, which allows the detection of outbreaks and the study of the evolutionary genetic diversity bacteria including *Salmonella enterica*. The comparison of WGS with traditional methods is essential, prior to implementing this technique in routine surveillance, which allows choosing the suitable technique that might be most useful for sub-typing bacterial species (Ferrari *et al*., 2017).Generally, there are presently two major approaches performing WGS, First generation sequencing (e.g., Sanger sequencing) and Next generation sequencing (NGS).

1.2.7.1 Next Generation Sequencing (NGS)

Next-generation sequencing systems have made substantial advances in DNA sequencing technologies, providing higher accuracy and considerably lower costs (Liu *et al*., 2012). The number of whole genomes stored in public repositories, such as the Online Genome Database (https://gold.jgi.doe.gov/, accessed on 15 July 2021), has grown exponentially due to next-generation technologies. Comparative analyses, such as pan-genomic analysis, have become possible with the great number of genomes available, particularly the prokaryotic genomes which drive gene discovery in the biomedical, biotechnological, and environmental fields (Metzker, 2010). Comparative genomics was used to examine intrinsic genomic features in other species. The virulence mechanisms in pathogenic organisms may be elucidated through the pan-genomic method by using multiple organisms of a single species or genera for the identification of similarities between genomes (Katiyar *et al*., 2020). This approach can map the occurrence of and establish phylogenetic relationships for evolutionary events (Ricke *et al*., 2018). Furthermore, comparative genomics can be employed in microorganisms with different habits to compare their gene repertoires and genome sizes as intracellular pathogens often encounter reduced evolution and gene loss (Merhej *et al*., 2009).

The development of NGS technologies has allowed to study and understand advanced characterization of bacterial genomes, deeply identification of taxonomy of complex microbiomes and outbreak analysis with higher resolution than First generation sequencing methods (Harris *et al*., 2013 ;Walker *et al*., 2013; Cao *et al*., 2017). In comparison with the traditional Sanger method, the NGS technologies have enhanced the sequencing and analysis speed, and they provide massively high-throughput from multiple samples as well as reduced the costs of WGS (Metzker, 2010 ; Mardis, 2011).

In addition to DNA sequencing, NGS technologies can be used for RNA sequencing (a.k.a where whole transcriptome sequencing, WTS), methylation sequencing (MeS), whole-exome sequencing (WES) and Candidate gene sequencing (CGS) (Pelizzola & Ecker, 2011; Kulski, 2016). Clinically, NGS allows the whole genome sequencing in single run for multiple bacterial pathogens from different patients or from multiple species present in an individual patient (Deurenberg *et al*., 2017). Therefore, this technology is widely used for infection control and prevention processes in the clinical microbiology laboratories (Zhou *et al*., 2016). Regarding NGS technology, there are presently two major technologies: 1- Short read sequencing technologies (a.k.a Second generation sequencing) 2- long read technologies (a.k.a Third generation sequencing).

1.2.7.1.1 Second Generation Sequencing

Second generation sequencing approach provides massively parallel sequencing with a reduced cost and higher accuracy data (Goodwin *et al*., 2016). These approaches are emerged as a typical tool for clinical use in population level studies and in variant discovery for surveillance of infectious disease (Van Dijk *et al*., 2014 ; Ardui *et al*., 2018) . The core principle of second-generation sequencing technology is mainly depending on chemical reaction and optical detection processes (Suwinski *et al*., 2019).

With more details by Kulski (2016), this technology performs parallel sequencing of random fragmented pieces of genomic DNA or cDNA without cloning step via a foreign host cell that is needed in first generation sequencing. In contrast to first generation sequencing, the fragmented pieces of DNA or cDNA are ligated to linker or adapter sequences to construct template libraries. These libraries amplified on a solid surface or on beads and then insulated within arrays or tiny emulsion droplets and Nucleotide identification is performed by luminescence detection or by electrical charge detection. They generate billions of nucleotide sequences within each single run, where very large datasets are produced after sequencing each genome in multiple times (Besser *et al*., 2018).

Second generation sequencing technology has different commercial platforms that differ markedly in terms of their chemical reaction, output length, accuracy and cost (Buermans & Den Dunnen, 2014). These technologies included: 1) pyrosequencing (454/Roche), this technology depending on pyrosequencing immobilize DNA on beads and

computationally detection of pyrophosphate (PPi) molecule that is enzymatically released after incorporation an un-labelled nucleotide into the newly synthesized strand chain (Margulies et al., 2005; Mardis, 2008) 2)Sequencing by Oligonucleotide Ligation and Detection (SOLiD/Life Technologies), it is based mainly on nucleotide sequencing by ligation of a fluorescently labeled DNA with the universal primer to identify the nucleotide position in each sequencing cycle (Margulies *et al.*, 2005) ;
3) semiconductor sequencing (Ion Torrent), this technology rely on the electronically detection of the nucleotide sequences inside microchip by detection of the pH changes after incorporate new nucleotide (Wang *et al.*, 2014); 4) Sequencing by synthesis Illumina (SOLEXA), Illumina technology is based on sequencing by synthesis of the complementary strand to the adaptor attached on a glass solid phase surface and detection of terminator nucleotides by fluorescence(Bentley *et al.*, 2008).

The Illumina platform represents the most successful sequencing system and currently occupies a major part of NGS technologies with more than 70% dominance of NGS market (Shendure *et al.*, 2011 ; Harris & Okoro, 2014). Industrially, Illumina provides multiple instruments: MiniSeq, MiSeq, HiSeq, NextSeq and NovaSeq, where they are varied with throughput and read lengths (Bharagava *et al.*, 2019). The MiniSeq and MiSeq machines generate low to mid throughput for each sample, inexpensive instrument prices, and are represent a reasonable choice for small public health laboratories, whilst, the extremely expensive HiSeq, NextSeq and NovaSeq instruments generate much higher throughput and reduced the per sample cost, and are therefore more suitable for large sequencing facilities (Besser *et al.*, 2018). However, All Illumina platforms have commonly been used for investigating of pathogens during infectious disease outbreaks (Raghavendra & Pullaiah, 2018).

The workflow of Illumina platform includes several steps:1) extraction of genomic DNA from sample; 2) Preparation of library, which ordinarily involves fragmentation of genomic DNA randomly and the ligation of adaptors; 3) Amplification of DNA fragments by Bridge PCR strategy, where this method amplifies the DNA fragment that have adaptors attached to complementary primers on the glass surface of the flowcell, this method generates a cluster of amplicons; 4) Automated sequencing and fluorescently detection of reversible-terminating nucleotides, as shown in Figure (1-1) (Buermans & Den Dunnen, 2014).

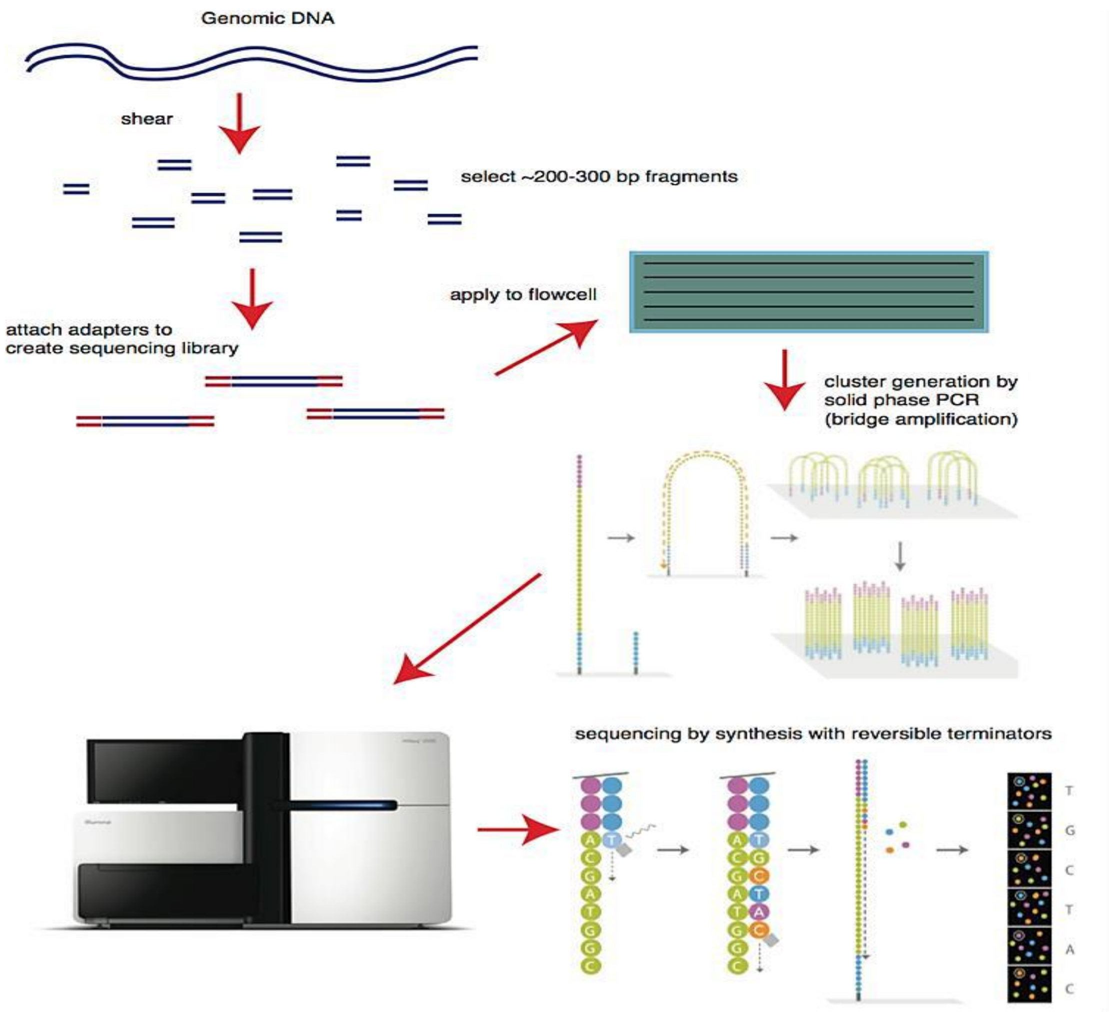


Fig. (1-1): An overview of Illumina sequencing technology

1.2.7.1.2 Third Generation Sequencing

Third-generation sequencing technologies have designed to address several limitations emerged in Second-generation sequencing technologies such as PCR amplification of the templates step, where this technology is characterized by sequencing of only one DNA molecule, i.e., the optic sensors are highly sensitive for fluorescently based detection of one nucleotide insertion (Méndez-Vilas & Teixeira, 2010). Directly sequencing of single DNA molecules in these technologies reduced low error rates, GC bias, intensity averaging, phasing alleles problems(Eid *et al*., 2009; Schadt *et al*., 2010). Even though third-generation sequencing technologies offer high accuracies, detection of epigenetic modifications, isoforms discovery and in applications of *de novo* genome assembly, they still have limited utility in the clinical setting compared with Short read sequencing technologies due to the low throughput and expensive cost(Goodwin *et al*., 2016; Suwinski *et al*., 2019).

Commercially, there are three providers for single molecule long read sequencing technology varying in their strategies: 1- Single-molecule realtime sequencing (SMRT®) by Pacific Biosciences, which it uses fluorescent nucleotides introduced into the reaction wells of their SMRT cell; 2- Nanopore sequencing (MinION) by Oxford Nanopore Technologies, these platforms guided DNA into nano-pores and detection of type of nucleotide is occurs by using ionic current sensing; 3- HeliScope sequencing system by Helicos BioSciences, this system performs single DNA molecules sequencing by synthesis and detects nucleotides that incorporated into the growing strand by using a highly sensitive fluorescence image system (Thompson & Steinmann, 2010 ; Bayley, 2015; Rhoads & Au, 2015).

Materials

&

Methods

2 Materials and methods:

2.1 Materials

All devices; laboratory tools; chemical materials and reagents; culture media; molecular and diagnostic Kits; used in this study have been explained in the following **tables: (2-1), (2-2), (2-3), (2-4)** and **(2-5)**, respectively.

2.1.1 Laboratory devices

The main devices and instruments used throughout this study are listed in **Table (2-1).**

Table (2-1) Laboratory devices

No	Instrument	Company	Country
1.	Autoclave	Stermite Olympus A&B	Japan
2.	Centrifuge	Hettich	Germany
3.	Cooling box	Ningbo	China
4.	Digital camera (phone camera)	Samsung	Korea
5.	Distillator	GFL	Germany
6	Freezer	Aucma	China
7.	Gel electrophoresis	Cleaver	USA
8.	Hood	Labogene	Denmark
9.	Incubator	Memmert	Germany
10.	Light microscope	Novel	China
11.	Nanodrop	Thermo Scientific™	USA

12.	Oven	Memmert	Germany
13.	Burner	Amal	Turkey
14	PCR thermocycler	Prime	UK
15.	Vitek 2 system apparatus	Biomerieux	France
16.	Refrigerator	Concord	Lebanon
17.	UV-transilluminator	Bioneer	Korea
18.	Vortex	Ohaus	USA
19.	Water bath	Memmert	Germany
20	Sensitive scale	Ohaus	USA

2.1.2 Laboratory instruments and tools

The main tools and instruments used throughout this study are listed in **table (2-2).**

Table (2-2) Laboratory tools

No	Instrument	Company	Country
1.	1000ul Aerosol Barrier+ Tips	Promega	USA
2.	10ul Aerosol Barrier+ Tips	Promega	USA
3.	200ul Aerosol Barrier+ Tips	Promega	USA
4.	Conical centrifuge tubes 30ml	Alfa	Jordan
5.	Conical flasks	Jlassco	India

6	Cover slide	Hirshman	Germany
7.	Cylinders	Jlassco	India
8.	Glass slides	Leitz	Germany
9.	Disposable Petri dishes	Grenier	Germany
10.	Micropipettes2-20µl,5-50µl,100-1000µl	Eppendorf	Germany
11.	Flipper rack 64 well	Promega	USA
12.	Loop	Shndon	England
13.	Medical cotton	Hygiene Limited	Jordan
14.	Medical gloves	Broche	China
15.	PCR tubes 1.5 ml (Eppendorf)	Biobasic	Canada
16.	PCR tubes 2.5 ml (Eppendorf)	Biobasic	Canada
17.	Plastic test tubes 10ml	DMD-DISPO	Syria
18.	Screw capped bottles 30 ml	DMD-DISPO	Syria
19.	Wooden sticks	Supreme	China

2.1.3 Chemical and Biological Materials:

The main chemical materials that used in this study are listed in Table (2-3).

Table (2-3) Chemical Materials

No	**Chemicals**	**Company/Country**
1.	Agarose	Promega / USA

2.	Absolute ethyl alcohol	Fluka / Germany
3.	DNA ladder marker	Promega / USA
4.	Loading dye (orange blue), Agarose, Master mix	Promega / USA
5.	Glycerol	Fluka / England
6.	Gram Stain Kit	Syrbio / Switzerland
7.	Hydrogen peroxide	Fluka / England
8.	Oxidase	Himedia / India
9.	Nuclease free water (1.25) ml	Promega / USA
10.	Tris EDTA (TE)	Bio Basic / Canada
11.	Tris-Borate-EDTA (TBE10X) buffer	Bio Basic / Canada
12.	Nucleic acid staining solution (Green Star$^{\text{TM}}$).	Bioneer / Korea

2.1.4 Culture Media:

The media have been used for culture in this study are listed in **table (2-4)**.

Table (2-4):- Culture media and their manufacturer.

No.	Culture Media	Manufacture / Origin
1.	Brain Heart Infusion Agar	BBL /France
2.	Brain Heart Infusion Broth	BBL /France
3.	Buffer pepton water	Himedia / India

4.	Kliglar Iron Agar	Himedia /India
5.	MacConky Agar	Himedia /India
6	Methyle red –vogous proskauer broth	Oxoid /UK
7.	Mueller-Hinton Agar	Mast /UK
8.	Nutrient Agar	Himedia /India
9.	Nutrient Broth	Himedia /India
10.	Salmonella Shigella agar	Himedia/India
11.	Simmon Citrate Agar	Himedia /India
12.	Tetrathionate broth	LlLab Mlimited/UK
13.	Urea agar base Christensen	Mast /UK
14	Xylose Lysine Deoxycholate Agar	Oxoid /UK

2.1.5 Commercial Kits:

The commercial kits used in the present study in the **table (2-5).**

Table (2-5) Commercial kits used in the present study

No.	***Type of Kit***	***Company/Country***
1.	DNA extraction kit	Geneaid / UK
2.	DNA ladder 2000bp , 3000bp	Promega / USA
3.	Green master mix	Promega / USA

4.	Primers	Microgen / Korea
5.	VITEK 2 AST-GN	Biomerieux/France
6.	VITEK 2 Gram Negative Identification Card (GN)	Biomerieux/France

Table (2-6): DNA extraction kit (Geneaid/UK).

Materials	
1- GT buffer	30 ml
2- GB buffer	40 ml
3- W1 buffer	45 ml
4- Wash buffer	25 ml +100 ml Ethanol
5- Elution buffer	30 ml

In addition to Proteinase K, Absolute ethanol and RNase A.

The kit contents were stored at 22-25ºC.

Table (2-7): Master Mix Used in PCR(promega/USA).

Materials
1-DNA polymerase enzyme (Taq)
2-dNTPs (400 µm dATP, 400 µm d GTP, 400 µm dCTP, 400 µm dTTP)
3-$MgCl_2$ (3mM)
4-reaction buffer (pH 8.3)

Table(2-8) DNA ladder

Materials
1-Ladder consist of 11 double-stranded DNA with size 100-2000bp. 2-Ladder consist of 13 double-stranded DNA with size 100-3000bp. 3-Loading dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA).

2.2 Methods

2.2.1 Sterilization Methods

A- Sterilization of the culture media by autoclave at 121ºC and pressure 15 psi for 15 minutes.

B- Sterilization of the glasses by dry heat in electric oven at 180ºCfor 2 h.

C- Sterilization of urea solution by Millipore filtration

2.2.2 Preparation of Reagents and solutions

2.2.2.1 Catalase Reagent

Hydrogen peroxide (3%) was prepared from stock solution in a dark bottle and it has been used for detection of the ability of the isolates to produce catalase enzyme (M Tille, 2017).

2.2.2.2 Oxidase Reagent

Oxidase reagent has been prepared by dissolving 0.1 gm. of Tetramethyl p-phenyl diamine- dihydrochloride in 10 ml of distilled water and it must prepared freshly in a dark bottle (M Tille, 2017).

2.2.2.3 Urea solution

Urea solution has been prepared by addition 40 gm. of urea crystal to 100 ml of D.W and then sterilized by filtration with Millipore filter paper (pore size 0.45µm).the suspension was poured in sterilized container and stored at 4◦C until it using (Joanne, *et al.*, 2008).

2.2.2.4 Iodine solution

Iodine solution prepared by dissolved (5 gm. of potassium iodide plus 6 gm. of iodine crystals) in 20 ml of D.W. (Macfaddin, 2000).

2.2.2.5 Agarose Gel:

The agarose gel was prepared according to the method of (P. Y. Lee *et al.*, 2012) by adding 1gm agarose to 100 ml of 1x TBE buffer. The solution was heated to boiling using a Bunsen flame. At 30 s intervals, the flask was removed and the contents was swirled to mix well. Repeat until the agarose has completely dissolved., the solution was allowed to cool down within 50-60^{o} C, and mixed with (5µl/ml) nucleic acid staining solution (Green Star^{TM}).

2.2.3 Preparation of cultural media:

2.2.3.1. Tetrathionate broth

This selective broth for isolation of *Salmonella* species, recommended by America health association was prepared by dissolving (46 gm.) of tetrathionate broth media in 1 letter of deionized water, heated to boiling with frequent agitation to completely dissolve the medium (over heating may destroy the selectivity of the medium), then cool to 47ºC and then add (20) ml of iodine solution that prepared previously (Macfaddin, 2000).

2.2.3.2 Urea agar medium

The ability of *Salmonella* spp.to produce urease enzyme is examined by This medium, it is prepared by dissolving (24 gm.) from urea agar base in (950 ml) of distilled water, and the pH must adjusted to 6.8 then the medium sterilized by autoclaving and cooled to 50ºC; then added fifty ml of 40 % urea solution prepared previously by Millipore filtration The Urea agar medium has been distributed in 5 ml amount as slant in sterile tubes in a slop form (Macfaddin, 2000).

2.2.3.3 MacConkey agar

This medium used to isolate and differentiate between bacteria which ferment and non-ferment lactose, prepared by weight (51.5 gm.) of medium , dissolving in (1 L.) of distilled water, heated to boiling with frequent agitation to completely dissolve the medium and sterilized by autoclave, then cooled to 55ºC and poured into petridishes ,this petridishes incubated in an incubator at 37ºC for 24 hours to remove any contaminated medium (Macfaddin, 2000).

2.2.3.4 Brain heart infusion agar

This medium prepared like (MacConkey agar) but the amount of this medium is (52 gm. /1L).

2.2.3.5 Brain heart infusion broth

This medium using to activate, grow and as stock culture for isolates *Salmonella*; it is prepared by dissolving (37 gm.) of medium in (1L.) of distilled water, and adding 20 % glycerin, then pouring to sterile test tubes and sterilizing by autoclave (Macfaddin, 2000).

2.2.3.6 Buffer peptone water (BPW)

This medium using as pre-enriched media for *Salmonella* isolation and using as a transport medium and to detect the ability of these bacteria to form the indole ring from tryptophan. It is prepared by dissolving (15 gm.) of medium in (1L.) of distilled water, then distribution into sterile test tubes and sterilizing by autoclave (Macfaddin, 2000).

2.2.3.7 Simmon's - citrate medium

Simmon citrate agar was made by dissolved 24.28 gm of medium in 1000 ml of distilled water to make this medium, after that, it was sterilized in an autoclave at 121°C for 20 minutes. Simmon's Citrate medium was used to test bacteria's capacity to use citrate as their only carbon source (Macfaddin, 2000).

2.2.3.8 Kligler Iron medium (KI)

This medium used for the presumptive identification of *Salmonella*; it is prepared by dissolving (55 gm.) of medium in (1L.) of distilled water and sterilized by autoclave, then the medium is distributing in 5 ml amount as slant in sterile tubes, and put in a slant position (Macfaddin, 2000).

2.2.3.9 *Salmonella–Shigella* agar (S.S agar)

This medium used for isolation and identification of *Salmonella* spp. and *Shigella* spp., it is prepared by dissolving (63 gm.) of medium in (1L.) of distilled water, heated to boiling with frequent agitation to completely dissolve the medium, then cooled to 55ºC in water bath and poured into petridishes, then incubated in an incubator at 37º C for 24 hours to remove any contaminated medium (Macfaddin, 2000).

2.2.3.10 Xylose-Lysine Deoxycholate agar (XLD)

This medium is used to isolation and identification of *Salmonella* spp. from stool samples, it is prepared like (SS agar) but the amount of this medium is (53 gm. /1L.).

2.2.3.11 Brilliant green agar

This medium used to isolation and identification of *Salmonella* spp. and it is prepared like (SS agar) but the amount of the medium is (29 gm. /1L.).

2.2.3.12 Nutrient agar

This medium prepared like MacConkey agar medium but the amount of this medium is (28 gm. /1L.).

2.2.3.13 Methyl red Vogues Proskauer media (MR-VP)

Methyl red Vogues Proskauer media (MR-VP Medium) or (Glucose Phosphate Broth) is recommended for perforation of the Methyl Red and Voges-Proskauer tests in differentiation of the coli-aerogenes group. it is prepared by suspend 17 gm. in 1L.of distilled water and heated if necessary to dissolve the medium completely then distribute in test tubes in 10 ml amounts and sterilize by autoclave (Macfaddin, 2000).

2.2.3.14 Maintenance medium

Maintenance media for bacterial isolates; the bacterial isolates have been preserved on brain heart infusion agar slant at 4 ºC. The isolates have been maintained monthly during the study by culturing on new culture media. For long preservation, brain heart infusion broth supplemented with 20% glycerol has been used and the isolates have been preserved frozen (-20 ºC) for long term (several months) (Fawole & Oso, 2004).

2.2.4 Subjects of the Study:

A total of 200 clinical stool and blood specimens were collected from patients in the stages of all ages and both sexes suffering from watery diarrhea or with (mucus , pus , little blood or no) and blood specimens obtained from patients with typhoid fever were admitted in three hospitals of Babylon Governorate: the Imam Al-Sadiq Teaching Hospital, Al-Hillah Surgical Teaching Hospital and Babel Teaching Hospital for Women and Children during a period of four months (from February 2022 to May 2022).

2.2.4.1. Study Design

A Cross-sectional study was designed that include 200 clinical stool specimens, obtained from patients with diarrhea and blood specimens obtained from patients with typhoid fever , attended to the Imam Al-Sadiq Teaching Hospital , Al-Hillah Surgical Teaching Hospital , and Babel Teaching Hospital for Women and Children during a period of four months from February to May 2022. As explained in **figure (2-1)**

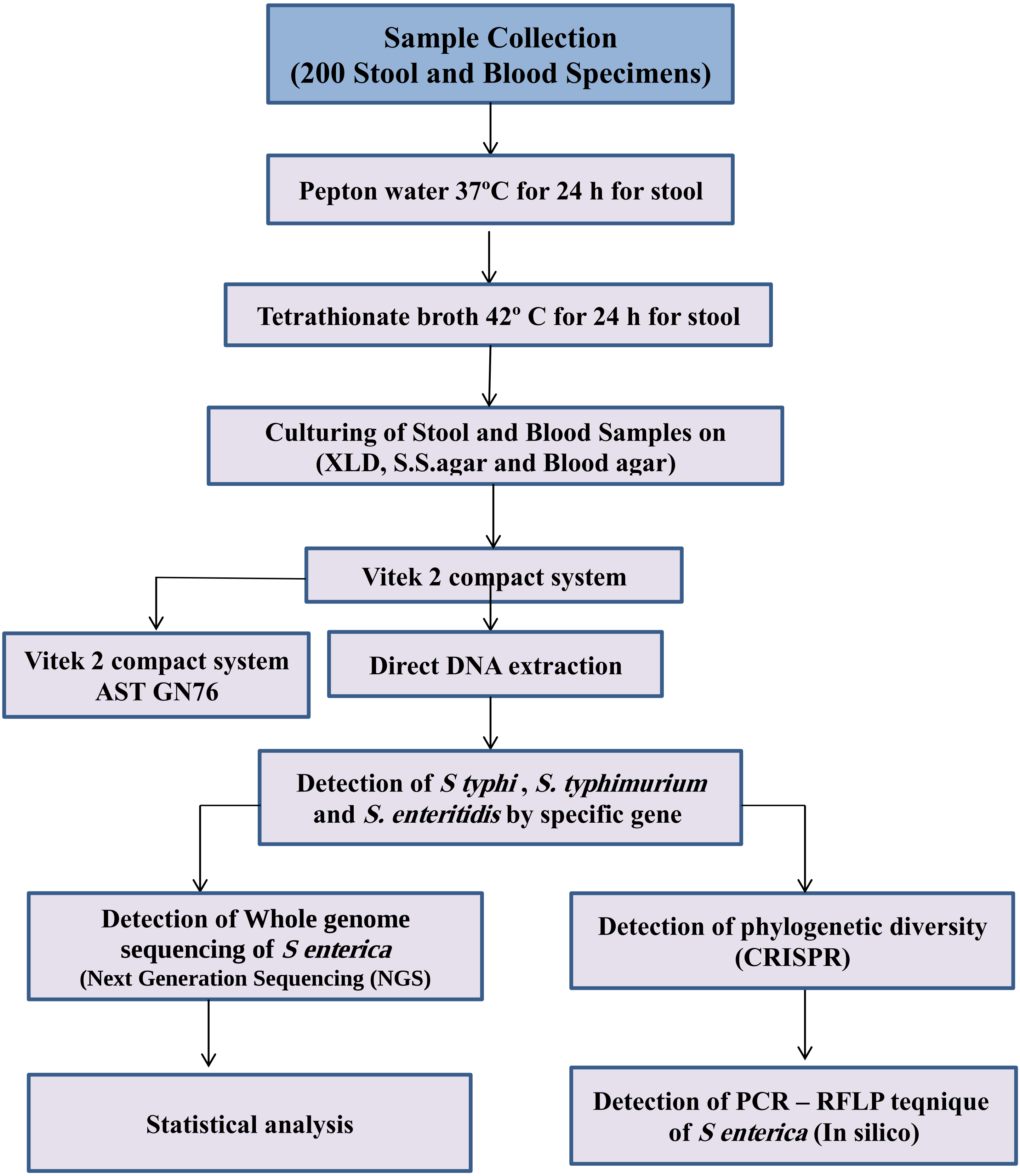


figure (2-1) Experimental design

2.2.4.2 Ethical approval:-

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. Verbal consent was taken from each patient or his parents before sampling. Investigative standards were rigidly preserved, primarily concerning confidentiality. Moreover, this study was undisclosed, participation of patients was optional, and verbal consent was received before data uptake process was started. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee (at College of Medicine University of Babylon).

2.2.5 Isolation and identification of *Salmonella enterica*

2.2.5.1 Samples collection

Two hundred fecal specimens were collected from Persons in the stages of all ages and both sexes suffering from watery diarrhea with (mucus , pus , little blood or no) and blood specimens obtained from patients with typhoid fever in the Imam Al-Sadiq Teaching Hospital , Al-Hillah Surgical Teaching Hospital and Babel Teaching Hospital for Women and Children in Babylon province during the period from February 2022 to May 2022. Fecal sample (1gm) were put immediately in a sterile tube contained buffered peptone water, and transmitted immediately to the laboratory of Collage of Medicine in Babylon university with cooling box (Cherneck & Berger, 2008).

Five ml of blood sample were immediately inoculated under complete aseptic conditions into in bottle contain 50 ml of Brain Heart Infusion broth, incubated at 37° C, and examined daily for up to seven days. Subcultures of blood culture broth were streaked on Xylose Lysin Deoxycholate agar (Cappuccino & Welsh, 2019).

2.2.5.2. Exclusion criteria

More than twenty cases are excluded from the study due to the absence of study criteria when patient use antibiotics after taken the history of patients.

2.2.5.3 Isolation of *Salmonella enterica* (WHO, 2010)

3.2.5.3.1 Pre-enrichment and enrichment

The buffered peptone water that contained the fecal sample was incubated into at 37ºC for 24 hours, and then culture in tetrathionate broth and incubated 42º C for 24 hours.

2.2.5.3.2 Culturing of samples

Spread 10 µl (loop full) from the inoculated and incubated Brain Heart Infusion broth and tetrathionate broth on XLD plates and incubated at 37^{o}C overnight (18-24 hours). The plates were examined based upon colonial phenotyping. *Salmonella* suspected colonies on XLD aseptically transferred to Triple Sugar Iron agar slants, urea agar slant, Citrate agar, and MacConkey agar for further confirmatory diagnosis by VITEK 2 compact system, and molecular diagnosis using PCR technique.

2.2.5.3.3 Gram's stain

According to (Riedel *et al*., 2019) take one drop of D.W.by loop full and a single colony from nutrient agar was taken by a loop and spread on a clean slide then wait to dry and fixing by heat, staining with gram stains and examined the bacterial cell under microscope (oil immersion).

2.2.5.3.4 Biochemical tests

Biochemical tests have been conducted according to (Cappuccino & Welsh, 2019) for identification of *Salmonella enterica*.

A. Kligler Iron Agar

Heavy inoculum was streaked over the surface of slope of Kligler agar and stab into the bottom, incubated aerobically at 37ºC for 24 hrs. Interpretation of the results was recovered by the change of color at surface and bottom, with or without H_2S production as follows:

Slant / Bottom	Color
Alkaline / Acid	Red / Yellow
Acid / Acid	Yellow / Yellow
Alkaline / Alkaline	Red / Red
H_2S	Black Precipitation
Gas	Bubbles

Result of Kligler iron agar of *Salmonella* spp. as follow: Alkaline/acid + H_2S + with Gas or without.

B. Catalase Test:

Catalase is an enzyme that catalase the release of oxygen from hydrogen peroxide. The nutrient agar medium was streaked with the selected bacterial colonies and incubated at 37C for 24 hrs, then the growth was transferred by the wooden stick and it was put on the surface of a clean slide ,a drop of (3%H2O2)was added. The Formation of gas bubbles indicates apositive result .

C. Oxidase Test:

This test depends on the presence of certain bacterial oxidases enzyme that would catalyze that the transport of electrons between electron donors in the bacteria and a redox dye (tetramethyl-p-phenylene-diaminedihydrochloride),the dye was reduced to a deep purple color.A strip of filter paper was soaked with a little freshly made reagent ,and the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep purple color which appeared within 5-10 seconds .

D. Lactose fermentation

Lactose fermentation is examined by culturing a single colony of suspected *Salmonella* suspected on MacConky agar then incubated at 37ºC for 24 hrs, rosy or pink colony is indicate to lactose fermentive while pale colony is indicate as non-fermentive bacteria.

E. Urease test

This test was done by culturing suspected *Salmonella* isolates on slant of urea agar that prepared previously, incubated at 37ºC for 24 hrs and if a result is converted color of media from yellow to pink color, it considered as positive result.

F. Indole test

It was done by inoculating peptone water by suspected *Salmonella* isolates and incubating at 37ºC for 24 hrs, then adding 0.5ml of Kovac's reagent and mixing gently, positive result appeared as the red ring in surface.

G. Citrate utilization test

It was done by culturing suspected *Salmonella* isolates on slant or petridishes of Simmon's –citrate agar then incubating at 37ºC for 48 hrs and a positive result is observed by converting the color of media from green to blue color.

H. Motility test

This test done by stapping suspected *Salmonella* isolates on Semi- solid media that prepared previously then incubated at 37ºC for 24 hrs.

2.2.6 Identification of the test organism also done by Vitek 2 Compact automated system (Pincus, 2010)

2.2.6.1 Sample processing in Vitek 2 compact: for *S.enterica*

2.2.6.1.1 Sample Preparation:

1- All the cultures had to be 18 to 24 hrs. old before testing on the machine.
2- The Vitek 2 compact Cassette was selected with labelled barcode defining cassette no. and fit the polystyrene tubes in the cassette.
3- Three ml sterile Vitek Saline was taken in the polystyrene tubes provided.
4- The isolated colonies was selected from the selected plate and suspend in Vitek Saline with the help of a sterile loop.
5- A uniform suspension was made and Vortex it thoroughly.
6- The inoculums density was checked and adjust it with the use of the Densi checked and making it 0.5 Mc Farland Standard.
7- One hundred forty five µl for AST Gram negative bacteria was transferred and 280µl for AST in case of Gram positive bacteria.
8- The selected cards were then ready for inoculation.

9- The selected cards was put in the inoculums in selected polystyrene tubes.

10- All the inoculums in the cassette were ready to put inside the filling section of the machine. The time duration of insertion must not exceed 30 minutes after inoculating cards.

2.2.6.1.2 Sample Loading:

1. The cassette was loaded into the filler door.
2. On the user interface screen was pressed "Fill".
3. Filling took 70 seconds.
4. When filling was finished, the blue indicator light on the instrument flashes.
5. Cassette was entered into the Load door.
6. Barcodes were scanned and checked against the Main Virtual Cassette electronic work list. 7. Straws are sealed.
7. Cards were loaded into the carousel.
8. Discard cassette wasted when it was finished.
9. Flashing blue arrow on the instrument indicates loading was finished.

2.2.6.1.3 Entering Cassette Worksheet Information:

Cassette Worksheet information is entered into the system software according to the user‟s manual given by Biomerieux and is saved in the system. Results of identification and Antimicrobial Susceptibility of test organism obtained after 18 hours.

2.2.7 Molecular study

2.2.7.1 Extraction of genomic DNA from bacterial culture

Genomic DNA was extracted from *Salmonella* isolates by using Geneaid Genomic DNA Purification Kit (UK) and done according to company instructions; the bacterial culture has been inoculated in 10 ml nutrient broth medium and incubated at 37ºC overnight in shacking incubator.

Step 1

Bacterial cells (up to 1 x 10^9) was transferred to a 1.5 ml microcentrifuge tube and Centrifuge for 1 min. at 14-16,000 x g then discarded the supernatant.

Step2

A 180 µl of GT Buffer was added then re-suspend the cell pellet by vortex or pipette and added 20 µl of Proteinase K (make sure ddH_2O was added). Then incubated at 60ºC for at least 10 minutes and during the incubation, invert the tube every 3 min.

Step 3

A 200 µl of GB Buffer was added then mixed by vortex for 10 seconds and incubated at 70ºC for at least 10 min. to ensure the sample lysate is clear and during the incubation ,then I inverted the tube every 3 min. At this time, pre-heat the required Elution Buffer (200 µl per sample) to 70ºCfor using in step (7) DNA Elution.

Step 4

A 200 µl of absolute ethanol was added and mixed immediately by shaking vigorously. If precipitate is appears, break it up as much as possible with a pipette then I placed the GD Column in a 2 ml Collection tube and transferred mixture (including any insoluble precipitate) to the GD Column and centrifuge at 14-16,000 x g for 2 min . Discarded the 2 ml Collection tube containing the flow-through and then placed the GD Column in a new 2 ml Collection tube.

Step 5

A 400 µl of W1 Buffer was added to the GD Column and Centrifuge at 14-16,000 x g for 30 seconds; discarded the flow-through then placed the GD Column back in the 2 ml Collection tube.

Step 6

A 600 µl of Wash Buffer (with ethanol) was added to the GD Column then centrifuge at 14-16,000 x g for 30 seconds , discarded the flow-through and placed the GD Column back in the 2 ml Collection tube and centrifuge again for 3 min. at 14-16,000 x g to dry the column matrix.

Step 7

The dried GD Column was transferred to a clean 1.5 ml microcentrifuge tube and added 100 µl of pre-heated Elution Buffer into the center of the column matrix then I waited stand for at least 3 min. to allow Elution Buffer to be completely absorbed and centrifuge at 14-16,000 x g for 30 seconds to elute the purified DNA.

2.2.7.2 Estimation of DNA Concentration

The extracted genomic DNA is checked by using Nanodrop spectrophotometer which measures DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260 /280 nm).

2.2.7.3 Dissolving and Preparation of Primers

All primer pairs used in this study were dissolved using TE Buffer, 1X (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA-Na_2. Firstly, the primer stock tube prepared and then the working solution would prepare from primer stock tube. According to the instruction provided by primer manufacturer (Bioneer / Korea) the TE buffer were added to get 100

Pico mole/ microliter concentration of primer stock solution. The working solution prepared from stock by dilution with TE buffer to get 10 Pico mole/ microliter and kept in -20 ºC.

2.2.7.4 The mixture of PCR reaction:

Amplification of DNA was carried out in final volume of 25 µl containing the following as mentioned in Table (2-9):

Table (2-9) Contents of the Reaction Mixture

No.	Contents of reaction mixture	Volume
1.	Go Taq Green master mix	12.5µl
2.	Upstream primer	1.5µl
3.	Downstream primer	1.5µl
4.	DNA template	3µl
5.	Nuclease free water	6.5µl
Total volume		25µl

2.2.7.5 Primer Sequences:

The oligonucleotide primers for all genes used in this study were obtained from previous studies and summarized in table (2-10).

Table (2-10) Primers sequences of all studied genes in this study with their amplicon size (bp) and reference.

Primer name	Primer sequence (5'-3')	Size bp	PCR condition	Reference
STM4497 F	**GGAATCAATGCCCGCCAATG**	**523**	**94 ºC 5min** **94 ºC 60Sec** **62 ºC 2min 35x** **72 ºC 2min** **72 ºC 10min**	(Shanmugasundaram *et al*., 2009)
STM4497 R	**CGTGCTTGAATACCGCCTGTC**			

SE1472298-2 F ***SE1472298-2 R***	**CTTGGAGAGCTGCGCTAAAG** **TAAGGCACCTCTCAACACTG**	**612**	**94 ºC 5min** **94 ºC 60Sec** **62 ºC 2min 35x** **72 ºC 2min** **72 ºC 10min**	(Ogunremi *et al*., 2017)
Tyv F ***Tyv R***	**GAGGAAGGGAAATGAAGCTTTT** **TAGCAAACTGTCTCCCACCATAC**	**614**	**95 ºC 5min** **95ºC 30Sec** **55 ºC 45Sec 30x** **72 ºC 2min** **72 ºC 8min**	(H. Levy *et al*., 2008)
***CRISPR-I* F** ***CRISPR-I* R** ***CRIRPR-II* F** ***CRIRPR-II* R**	**GCTGGTGAAACGTGTTTATCC** **ATTCCGGTAGATYTKGATGGAC** **AACGCCATGGCCTTCTCCTG** **CAAAATCAGYAAATTAGCTGTTC**		**94 ºC 5min** **94 ºC 50min** **50 ºC 1min 40x** **72 ºC 1min** **72 ºC 5min**	(Grissa *et al*., 2007)

2.2.7.6 Molecular detection of *Salmonella enterica* using uniplex PCR:

For Amplification of *Salmonella enteritidis*, *Salmonella Typhimurium* and *Salmonella Typhi*,, a Uniplex-PCR was done a total volume of 25 µl (Primers 1.5x2, DNA 3 µl, Master mix 12.5 µl, RNAse free water 6.5 µl). The primers were used for this reaction by using GoTaq® G2 Green Master Mix (Promega, USA) **(table 2-11)**. A 5 µl of the PCR products were loaded into 2% agarose gels in 1 X TBE, and run at 100 v in 1X TBE for 40 minutes. Images of the gels were captured using a gel documentation system. The PCR products for *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella typhi* were 612bp, 523bp and 614bp, respectively .

Table (2-11):- Uniplex PCR component to detection *of S. enterica* .

Component	**Amount**
RNAse free water	6.5 µl
Go Taq Green master mix	12.5 µl

F primer: *S. enterica*	1.5 µl
R primer: *S. enterica*	1.5 µl
DNA sample	3 µl
Total sample	25 µl

2.2.7.7 Amplification of CRISPR arrays:

CRISPR array amplification was performed according to Lertworapreecha et al., (2018)using primers specific for CRISPR-I (F_CR-I: 5’-GCTGGTGAAACGTGTTTATCC-3’;R_CR-I:5’-ATTCCGGTAGAT YTKGATGGAC-3’,) and CRIRPR-II (F_CR-II: 5’-AACGCCATG GCCT TCTCCTG-3’; R_CR-II: 5’-CAAAATCAGYAAATTAGCTGTTC-3’). This reaction by using GoTaq® G2 Green Master Mix (Promega, USA). The reaction mixture (25 mL) composes of (Primers 1.5x2, DNA 3 µl, Master mix 12.5 µl, RNAse free water 6.5 µl). Amplification condition was performed as described, initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 50 min, annealing at 50 °C for 1 min, primer extension at 72 °C for 1 min, and one cycle with a final extension step at 72 °C for 5 min. The amplification product was analyzed and visualized by 1.5 % agarose gel electrophoresis stained with a Ethidium bromide (Thermo Fisher Scientific, MA, USA).

2.2.7.8 CRISPR analysis:

All PCR products from positive samples were purified and analyzed by sanger sequencing method in Korea (Macrogen company), and all sequences were analyzed for CRISPR- I and CRISPR-II by CRISPRFinder program (Grissa *et al.*, 2007). The diversity of CRISPR-I, CRISPR-II and direct repeat were aligned using Bioedit (Hall, 2004) and MEGA7 program(S. Kumar *et al.*, 2018).

2.2.7.9 In silico PCR-RFLP

The in-silico PCR-RFLP of the CRISPR arrays were simulated by NEBcutter V2.0 (Vincze *et al*., 2003), using the *Bst*UI restriction enzyme (*Bacillus stearothermophilus* UI) which recognizes and cuts at specific sequence (CG/CG) along the CRISPR. Determination of the DNA size was also analyzed by the software (NEBcutter V2.0).

2.2.7.10 Detection of Amplified Products by Agarose Gel Electrophoresis:

The PCR amplification of products were analysed by Agarose gel electrophoresis using 1% agar rose gels prepared by dissolving 1 g of agarose (Paião *et al*., 2013) mixed with 100 ml of 10 x Tris - Borate EDTA (TBE) buffer (10ml TBE+90ml sterile distilled water) heated to boil on hot plate. The agarose gel was cooled down to 45°C where 5µl of Ethidium bromide stain.

The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was powered gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covering the surface of the gel, 5µl of DNA sample was transferred into the signed wells in agarose gel, and in one well we put the 5µl DNA ladder mixed with 1µl of loading buffer.

The electric current was allowed at 70 volt for 30 min. UV transilluminater was used for the observation of DNA bands, and gel was photographed using a digital camera.

2.2.7.11 Whole Genome Sequencing (WGS)

After extraction of Genomic DNA by Presto™ Mini gDNA Bacteria Kit supplied from Geneaid company, the extracted DNA subjected to quantification by NanoDrop instrument to estimate the DNA concentration according to manufacturing's instructions. In addition, the condition of the DNA was assessed by gel electrophoresis method to evaluate the presence or absence of DNA in the sample, where 1µl of DNA loaded to 1% agarose gel and run at 160V for 30min. Following this step, only successful samples were submitted to Macrogen company (Korea) for WGS (Paired-ends) using the Illumina NovaSeq 6000 platform. The resulted raw reads were processed by further bioinformatics tools.

2.2.7.12 Bioinformatics Analysis

In this analysis, all bioinformatics approaches that used to analyze the study sequences were relied on either using command-line tools and bioinformatics softwares on open-source operating system, Linux (Version: Ubuntu 18.04.3 LTS, Canonical Ltd., UK), or using web-based servers such as the Galaxy platform (https://galaxyproject.org) (Afgan *et al*., 2018).

Before WGS analysis, the raw data undergo quality control by FastQC (Version, 0.11.5) (Andrews, 2010) to evaluate quality of reads and calculating the basic statistics (such as total number of bases, reads and GC content). After quality control, raw reads were subjected to preprocessing steps to reduce biases in analysis by trimming out bases of low quality, adapter sequences and the Poly-G tail. Fastp (version 0.19.4) and Trimmomatic (version 0.36) tools were used for preprocessing steps (Bolger *et al*., 2014; Chen *et al*., 2018). The filtered raw data have undergone for further processing steps included Multiple genome alignment by Mauve (v2.4.0) (Darling *et al*., 2010); Annotation by Rapid Annotation using

Subsystem Technology, RAST (v 2 RASTtk) (Brettin *et al*., 2015); Mapping by Burrows-Wheeler Aligner BWA, (BWAMEM) (v0.7.17)(Li, 2013); Proteome comparison by PATRIC and The SEED Viewer (v2.0) (Wattam *et al*., 2017 ; Overbeek *et al*., 2014); Variants calling by SAMTools(Li, 2011); Variant annotation type and impact by SnpEff (v4.3t) (Cingolani *et al*., 2012).

Results

&

Discussion

3.1 Isolation and identification of *Salmonella enterica* :

3.1.1 Isolation of *Salmonella enterica* :

In the present study a total of 200 clinical stool specimens were collected during this study which obtained from patients in the stages of all ages and both sexes suffering from watery diarrhea or with (mucus , pus , little blood or no) and blood specimens obtained from patients with typhoid fever who admitted to three main hospitals of Babylon Governorate:

The Imam Al-Sadiq Teaching Hospital , Al-Hillah Surgical Teaching Hospital and Babel Teaching Hospital for Women and Children during the period from February 2022 to May 2022.

All sample were subjected to aerobic culturing on selective media and it was out of the total (200 specimens), 180 specimens showed positive bacterial culture , no growth was seen in other 20 specimens which indicate the presence of microorganisms that may be cultured with difficulty such as virus and other agent or may be due to difference in the size and nature of the specimens.

Among (180) positive culture , only 34 (17%) specimens show positive results on culture identified as *S. enterica* as shown in the table (3-1).

Table(3-1)prevalence of *Salmonella enterica* among other etiological agents associated with sample isolated.

No. Sample	Culture and Vitek 2 compact			Molecular diagnosis	
	S. enterica	Negative results	Other bacteria and fungi	Positive results	Negative results
200	34(17)%	20(10)%	146(73)%	25(73.53)%	9(26.47)%

Previous study obtained by (Al Jobouri , 2019) who found that (17.5%) were identified as carriers of *Salmonella enterica* on the basis of the results of the cultures of specimens. Also results obtained by (Andoh *et al*., 2017) who were found that (16.05%) were identified as carriers of *Salmonell enterica* .

The study done by Yousif & Harab (2011) in Thi-Qar hospitals, mentioned that *Salmonella* account at (11.17%) from diarrheal cases. Other study reported by (Hanan, 2016) was show that the prevalence of *Salmonella enterica* was 8%.

The variation of isolation rate of *S. enterica* between studies depends on several factors like virulence of isolates , health status of patients and effect of environmental conditions, isolation and identification techniques , social and cultural level of patients and may be due to differences in the size of samples.

Salmonellosis remained one of the three most common meat associated diseases in human (Cooper, 1994). The likelihood of infections with *Salmonella* spp. increases due to the animal products are easily contaminated with microorganisms and support their growth if not properly handled, processed and preserved (Norhana *et al*., 2010) and the disease caused by *Salmonellae* organisms is the most common and important zoonotic diseases (Commission & Committee, 2008).

On the other hand, the using of antimicrobial causes a transient increases the likelihood of infection on exposure to a foodborne pathogen, such as *Salmonella* spp. and decrease in an individual’s resistance to colonization with these non-commensal bacteria (Miriagou *et al*., 2003).

S. Enteritidis and *S. Typhimurium* are the most predominant isolated organisms in most *Salmonella* cases associated with the consumption of contaminated poultry, and beef products(Vose *et al*., 2011). Typhoid fever is a major public health problem where safe drinking water and sanitation are inadequate (Rahman *et al*., 2014). Contamination with *Salmonella* in poultry products can occur at multiple steps along the food chain, including production, processing, distribution, retail marketing, handling, and preparation (Dookeran *et al*., 2012).

3.2 The characterization of *S. enterica*

3.2.1 Culturing of bacteria

The identification of *S. enterica* depends on the cultural and biochemical characteristics (vitek 2 compact) and also microscopic patterns . The organisms are gram negative bacillus microscopically appear rod shaped non spore forming predominantly motile enterobacteria with cell diameters between about 0.7 and 1.5 µm, lengths from 2 to 5 µm, and peritrichous flagella (all around the cell body)(Fàbrega and Vila, 2013).

facultative anaerobic bacilli, like other members of the family Enterobacteriaceae, they produce acid on glucose fermentation; reduce nitrates to nitrite, and don’t produce cytochrome oxidase (Torrence & Isaacson, 2008).

The results of the biochemical tests showed that these isolates gave positive results for catalase while it is gave negative results for oxidase, urease, and indole tests and isolates gave positive results to citrate utilization test as shown in table (3-2).

Table (3-2) The most important traditional tests used in identification of *Salmonella enterica*

Test	Results
Xylose-Lysine Deoxycholate agar(XLD)	small, smooth, rounded, red in color with black center
Brilliant green agar(BGA)	small, rounded, translucent rosy
Salmonella-Shigella agar (S.S.Agar)	small, smooth, rounded, pale with black center
MacConkey agar	transparent, colorless colonies with no precipitated zone
Nutrient agar(NA)	small, smooth, rounded and pale
KI test	Red/Yellow with H_2S production with gas production
Catalase test	+
Oxidase test	-
Lactose fermentation	Non-lactose fermenter
Urease test	-
Indole test	-
Methyl red	+
Voges proskauer	-
Citrate utilization test	(+) for *S. typhimurium* and *S. enteritidis*, (-) for *S typhi*
Motility test	+

3.2.2 Direct rapid identification of *S. enterica* by Vitek 2 compact

The Vitek 2 Gram-Negative identification card (GN) is used for the identification of clinically-significant fermenting and non-fermenting gram- negative bacilli. The GN card is based on standardized biochemical methods and newly developed substrates measuring carbon source utilization, enzymatic activities and resistance (Pincus, 2010).

Identification of an organism is based on the characteristics of the data and knowledge about the organism and resulting reactions being analysed. The software compares the test set of reactions to the expected set of reactions of the organism or organism group. This forms part of the identification process(Pincus, 2010).

After identification of bacterial colonies on the conventional biochemical test as were summarized in table (3-2), the isolates were identified with Vitek2 compact auto analyzer system The results produced by the machine were analyzed using compact software.

In this study the identification of *S. enterica* isolates were confirmed by using Vitek2 system compact, which is the standard biochemical identification system for bacteria. The analytical profile index of this system has showed the probability rate of identification scored more than 95 % with excellent confidence, **as shown in appendix (1)**.

The vitek2 system provide on accurate microbial identification. phenotypic confirmatory tool, and it is the most automated platform available, with rapid results, and high degree of specificities with proved confidence, and minimal training time than with manual microbial identification techniques.

3.2.3 Molecular identification of *S. enteritidis* , *S. typhimurium* and *S typhi* by Uniplex PCR using specific primer.

The aim of this study was to develop a rapid molecular diagnostic test to identify and purify bacterial isolates based on the specific primer , comparison with traditional criteria and that they may be good conditions to be used as genotyping (marker) for confirmatory identification of *S. typhimurium* and *S. enteritidis* from diarrhea cases and *S typhi* from typhoid fever cases.

For *Salmonella enterica* ser. Enteritidis specific motifs were found in the target gene *SE1472298-2* , the primers flanking portion length 612 bp were selected (Ogunremi *et al*., 2017) . The target gene *STM4497* demonstrated specificity for *Salmonella enterica* ser. Typhimurium , the primers flanking region 523 bp were choosed (Shanmugasundaram *et al*., 2009). *Salmonella enterica* ser. Typhi specific motifs were found in the target gene O antigen synthesis *tyv* (*tyv*, previously called *rfbE*) gene were used in this study, the primers flanking portion length 614 bp were selected (H. Levy *et al*., 2008).

Confirmation of diagnosis for *Salmonella enterica ser.typhimurium* depend on *STM4497* , *Salmonella enterica ser. enteritidis* depend on *SE1472298-2* and *Salmonella enterica ser.typhi* depend on O antigen synthesis *tyv* in Uniplex PCR , the results revealed that only 25 out of 34 were positive for culture and biochiemical test, these 25(73.53) isolates were includes 13(52)% of *S. enteritidis* , 6(24)% of *S. typhimurium* and 6(24)% of *S. typhi* as shown in figures (3-1) , (3-2) & (3-3) .

S. enteritidis was most frequent identified from human cases the present study was correlated with study obtained by (Rodulfo *et al*., 2012) who were reported that *S enteritidis* was the most common.

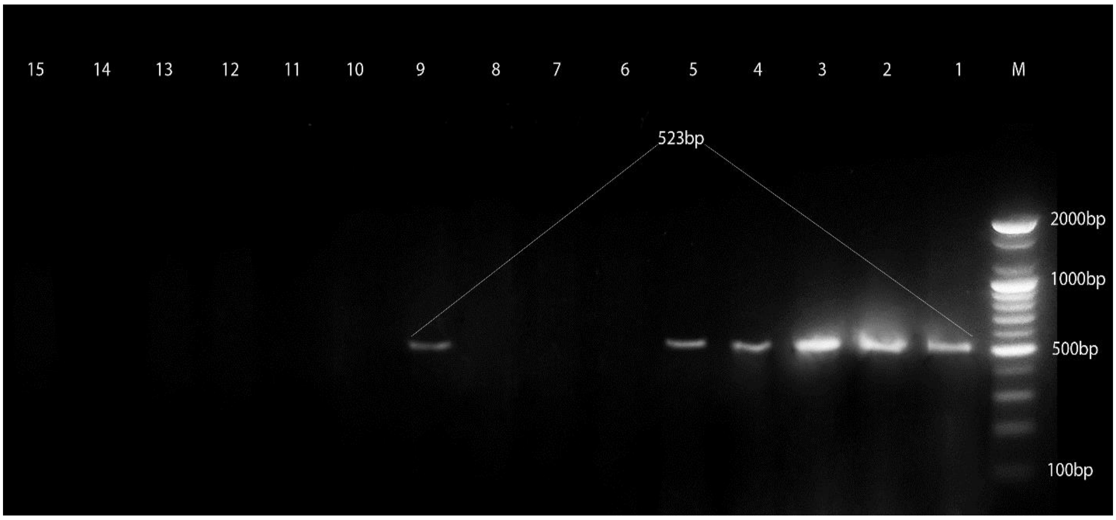


Fig. (3-1): 1% Agarose gel electrophoresis at 70 volt for 30 min for *Salmonella Typhimurium*- specific primer that generated 523bp amplicon, Lanes: 1-5 and 9 are positive, others are negative. M lane is DNA Ladder 100bp.

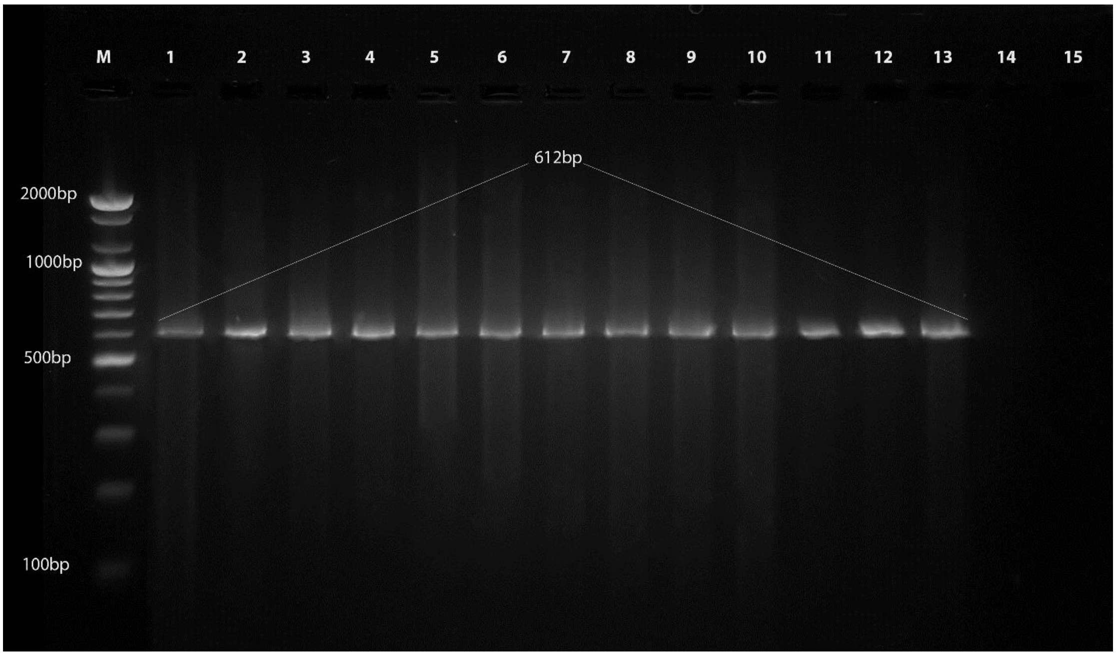


Fig. (3-2): 1% Agarose gel electrophoresis at 70 volt for 30 min for *Salmonella Enteritidis* - specific primer that generated 612bp amplicon, Lanes: 1-13 are positive, others are negative. M lane is DNA Ladder 100bp.

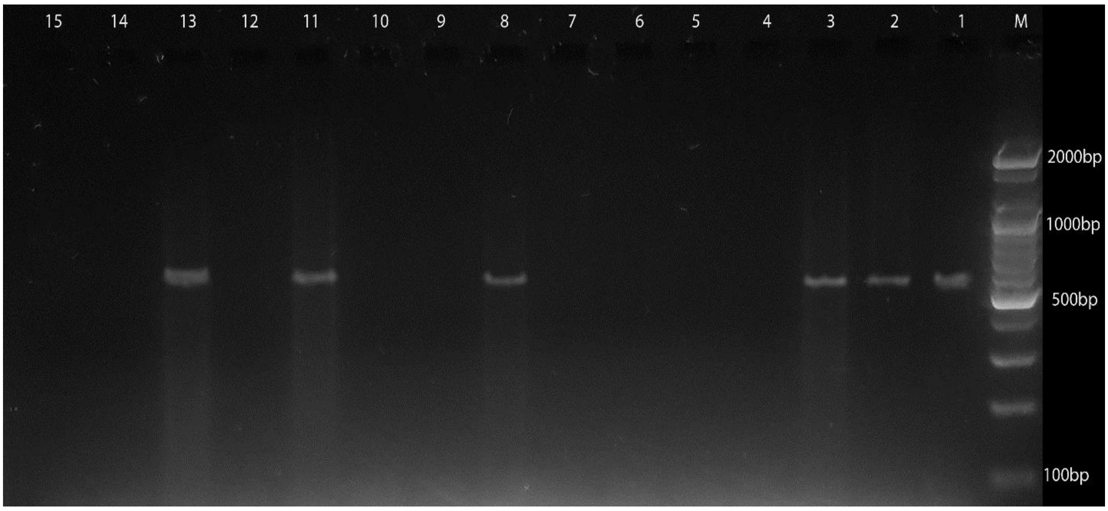


Fig. (3-3): 1% Agarose gel electrophoresis at 70 volt for 30 min for *Salmonella Typhi*- specific primer that generated 614bp amplicon, Lanes: 1-3, 8, 11, 13 are positive, others are negative. M lane is DNA Ladder 100bp.

The results was obtained in this study by molecular methods were 13 (52%) from *S. enteritidis* , 6 (24%) from *S. typhimurium* and 6 (24%) from *S. typhi* out of 34 specimens was positive results for *S. enterica*. Previous study results obtained by Heymans *et al*., (2018)who were found that (23%) were identified as carriers of *Salmonella enteritidis* and (32%) were identified as carriers of *Salmonella typhimurium* . While study by (Al Jobouri , 2019) who were found that 17 (70.83%) isolates *S. typhimurium* and 7 (29.16%) isolates *S. enteritidis*.

Other study by Al-atabi **,** (2021) shown that 10 (33.3%) from *S. typhimurium* and 8 (26.6%) from *S. typhi* .

Some molecular methods are introduced for rapid detection of *Salmonella* particularly those techniques with good reproducibility and rapidity. PCR and similar nucleotide-based methods have become potentially powerful alternative approaches in microbiological diagnostics because of their higher user-friendliness, rapidity, reproducibility, accuracy and affordability(Ranjbar *et al*., 2013).

Culture based methods are slow-they can take days between when patient first is seen by a doctor and time the result back from the clinical lab., for this reason, molecular method are much quicker than the culture based method ,so the (*SE147229* , *STM4497* & *tyv* gene) are a gold stander for identify *S. enterica* beyond phenotypic method.

However, this approach will enhance the accuracy, sensitivity specificity, special and cost effectiveness in the detection of *S. enterica* than culture technique and the PCR is the best choice for diagnosis of infection with *S. enterica* . However, molecular technique has over convectively methods, it can provide results in 24 hr .where as routine culture followed by biochemical test need 24-48hr .

The diagnosis of typhoid fever is made by ordinary culture methods and biochemical tests. However, the classic diagnosis method for typhoid fever requires at least 4 or 5 days for positive results. A rapid, alternative method is needed for the diagnosis of typhoid fever (Karami *et al.*, 2011). Molecular biology-based techniques including PCR assays have been reported for the rapid, specific, and sensitive detection of microorganisms in different clinical samples (Faik *et al*., 2014).

3.3 Antibiotic susceptibility test to *Salmonella enterica*:

Antibiotic susceptibility test (AST) is traditionally performed through the Kirby-Bauer disc diffusion assay. However, this method is laborious and prone to inconsistencies, subjectivity, and human error.The VITEK 2 system (bioMerieux) has revolutionized AST through its rapid and automated fluorescence-based technology that allows determination of minimum inhibitory concentration (MIC) by the analysis of growth kinetics of bacteria with antibiotics in test cards (Livermore *et al*., 2002) **as shown appendix (2).**

AST was done for all *Salmonella enterica* isolates . These isolates showed about 69% sensitive , 31% resistance towards antibiotics used in this study as show in table (3-3) .

Table (3-3):Percentage of antibiotics sensitive, intermediate and resistance by *Salmonella enterica* against 16 types of antibiotics by vitek2 compact according to CLSI, 2012 (n=25).

Antibiotics	**Sensitive (NO.)%**	**Intermediate (NO.)%**	**Resistant (NO.)%**
Ampicillin	(16) 64	-	(9) 36
Piperacillin/Tazobactam	(24) 96	(1) 4	-
Cefazolin	(3) 12	-	(22) 88
Cefoxitin	(3) 12	-	(22) 88
Ceftazidime	(21) 84	-	(4) 16
Ceftriaxone	(21) 84	-	(4) 16
Cefepime	(23) 92	(2) 8	-
Ertapenem	(23) 92	(2) 8	-
Imipenem	(25) 100	-	-
Amikacin	(2) 8	-	(23) 92
Gentamicin	(2) 8	-	(23) 92
Ciprofloxacin	(16) 64	-	(9) 36
Levofloxacin	(3) 12	-	(22) 88
Tigecycline	(25) 100	-	-
Nitrofurantion	(13) 52	(5) 20	(7) 28
Trimethoprim/Sulfamethoxazole	(25) 100	-	-

Highest rate of sensitive is seen to almost antibiotics used in present study which include Imipenem , Tigecycline and Trimethoprim/Sulfamethoxazole at rate (100%), Piperacillin/Tazobactam at rate (96%) , Ertapnem , Cefepime at rate (92%), while Ceftazidime and Ceftriaxone at rate (84%) as show in figure(3-4).

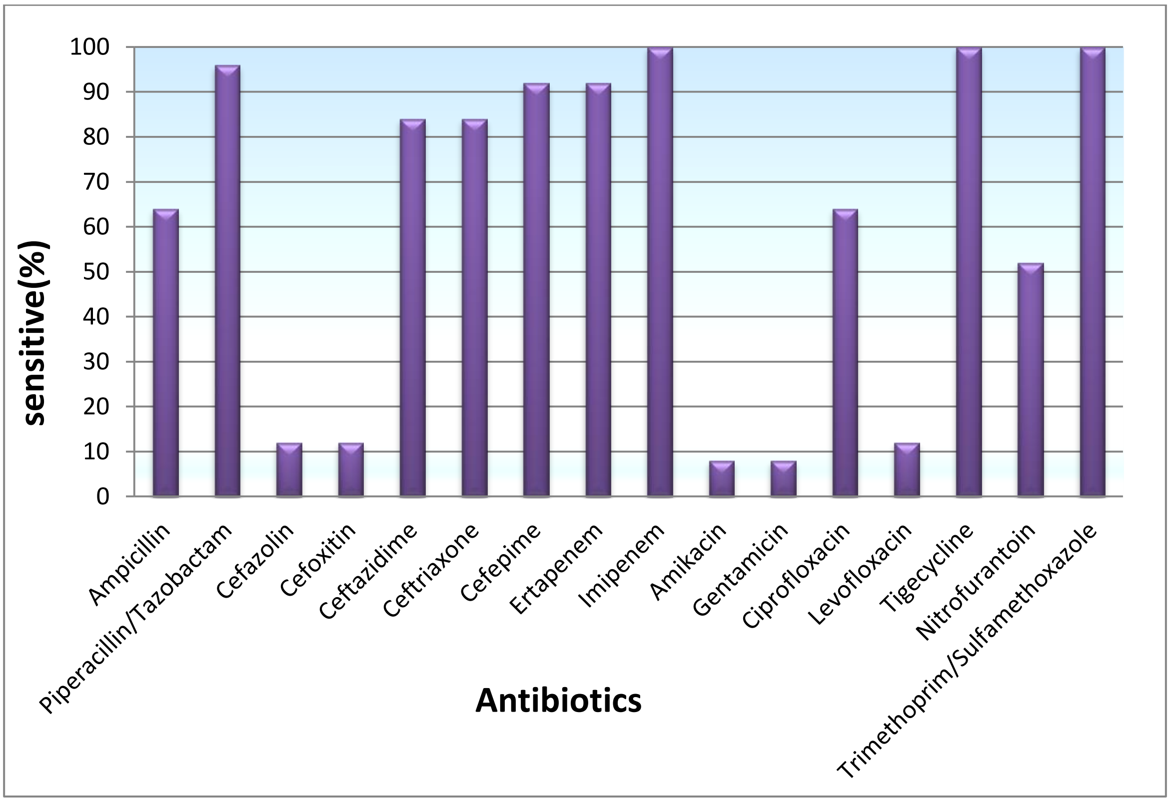


Figure (3-4): Show Antibiotic susceptibility of *Salmonella enterica*

The β-lactam antibiotics work by inhibiting the cell wall synthesis by binding and interfering with the structural cross linking of peptidoglycans thus preventing terminal transpeptidation in the cell wall. As a consequence it weakens the cell wall of the bacterium and finally results in cytolysis or death due to osmotic pressure (Lee *et al*., 2011).

In present study 100% of isolates were sensitiv to Imipenem, 92% of isolates were sensitive to Ertapenem and 96% of isolates were sensitive to Piperacillin/Tazobactam , study by (Ng & Rivera, 2014) who were found the percentages of antibiotics in *Salmonella enterica* isolates were 100% , 95% and 100% respectively . According to other study done by (Livermore *et al*., 2011) , ertapenem was the most active agent tested against members of the family Enterobacteriaceae including *Salmonella* spp. as compared to imipenem, cefepime, ceftriaxone, and piperacillin-tazobactam. In our study also found other antibiotic from the β-lactam antibiotics group is Ampcillin that showed 64% of isolates were sensitive to this antibiotic , Ng & Rivera, (2014) who were found percentage of antibiotic in *Salmonella enterica* isolates were 50% to Ampcillin .

On the other hand ,the present study also showed that *Salmonella* resistance to Ampicillin at rate 36% , study obtained by (Hanan, 2016) who found 33.3% resistance percentage for *Salmonella* isolates for Ampcillin.

However , Cephalosporins have a mechanism of action similar to other beta-lactam antibiotics and acts by inhibiting the peptidoglycan layer of the bacterial cell wall . In present study, 84% of isolates were sensitive to Ceftazidime and Ceftriaxone , 92% of isolates were sensitive to Cefepime . Dakorah (2014) who were found percentages of antibiotics susceptibility in *Salmonella enterica* isolates were 100% to Ceftazidime , Ceftriaxone and Cefepime.

On the other hand ,resistance to broad spectrum cephalosporins is mainly due to the organism’s production of extended-spectrum cephalosporinases. It has been reported that *Salmonellae* produce a variety of cephalosporinases enzymes; the genes encoding these extended-spectrum cephalosporinases are carried by conjugative plasmids, transposons, or integrons (Makanera *et al*., 2013; Su *et al*., 2013). In present study 88% of isolates were resistance to Cefazolin and Cefoxitin .However, Dakorah (2014) who were found percentages of antibiotics resistance in *Salmonella* isolates were 100% for cefazolin and cefoxitin .

Tigecycline is the first member of the glycylcycline class to be advanced into clinical trials and was approved by the US Food and Drug Administration (USFDA) as a parenteral agent for the treatment of intra-abdominal infections (Nathwani, 2015). Our study appeared that 100% of isolates were sensitive to Tigecycline , other study obtained by Ng & Rivera, (2014) who were found percentages of antibiotic in *Salmonella* isolates was 85% .

The stimulation use Trimethoprim- sulphamethoxazole results in the inhibition of sequential metabolic steps , the two components of this combination product have a similar antimicrobial spectrum. They operate synergistically because they independently inhibit different steps in the enzymic synthesis of tetrahydrofolic acid, an essential metabolic process in susceptible bacteria. The combination enhances the antibacterial efficacy of the individual constituents and impedes the emergence of resistance (WHO , 2021). The results of this study was 100% of isolates were sensitive to Trimethoprim-sulphamethoxazole .Study by (Kinney, 2019) who were found percentages of antibiotic in *Salmonella* isolates was 100% for Trimethoprim-sulphamethoxazole .

Highest rate of resistance is seen to almost antibiotics used in present study which include Cefazolin , Cefoxitin , Amikacin , Gentamicin and Levofloxacin was 88% , 88% , 92% ,92% and 88% respectively.

Furthermore , aminoglycosides have a broad antimicrobial spectrum; they inhibit protein synthesis and/or alter integrity of the bacterial cell membrane (Vakulenko & Mobashery, 2013). In our study showed that 92% of isolates were resistance to Amikacin and Gentamycin, however

Dakorah (2014) who was found the percentages of antibiotics resistance in *Salmonella enteric* isolates were 100% for Amikacin and gentamicin .

In the present study other antibiotic from group that inhibit protein synthesis(30S ribosome) is nitrofurantion showed sensitive percentage was 52% and resistance percentage was 28% , study by (Hanan, 2016)who was found *Salmonella* isolates were sensitive for this antibiotic 50% and resistance for the same antibiotic 25%.

Fluoroquinolones (e.g., ciprofloxacin) have become a mainstay for treating severe *Salmonella* infections in adults. Fluoroquinolone resistance in Salmonella is mostly due to mutations in the topoisomerase genes, but plasmid-mediated quinolone resistance (PMQR) mechanisms have also been described (Sjölund-Karlsson *et al*., 2014). In our study ciprofloxacin showed resistance percentage 36% to *Salmonella* isolates . Howover study by (Hanan, 2016) who found 25% resistance percentage for *Salmonella* isolates for ciprofloxacin.

This spread of antimicrobial resistance through the food chain is regarded as a major public health issue(Lynch *et al*., 2016). The appearance of both plasmid mediated antibiotic resistant against conventional anti- *Salmonella* drugs and chromosomal resistance to quinolones and fluoroquinolones has reduced therapeutic options for *Salmonella enterica* in humans(Elmadiena *et al*., 2013).

Current study showed that 88% of isolates were resistance to Levofloxacin ,study by (Pribul *et al*., 2017) who obtained percentage 30.2% to levofloxacin resistance for *salmonella* isolates.

The level and extent of resistance among *Salmonella* varies in different geographical locations. Nevertheless, there is a correlation between the length of time antimicrobial agent has been used and its corresponding resistance(McDermott, 2015).

The use of antibiotics is fundamental for controlling the epidemiology of *salmonellae*, but the emergence and widespread of multidrug-resistant strains to antibiotics responsible for gastroenteritis keeps worsening the situation (Dougnon *et al*., 2017).

Antibiotics resistance may be due to regional and/or population differences between studies and as some serotypes have unique epidemiological features that may lead to invasive diseases and might occur in specific populations or geographic areas or be associated with antibiotic resistance (Dutra *et al*., 2014).

Iraq is one of the developing countries where antibiotics sold over the counter, an attitude that encourages self-medication. In other hand, remarked that during period of time a group of antibiotics become more usage than other antibiotics without susceptibility tests which may lead to variability in resistance to these antibiotics.

Patients with invasive Salmonellosis require antimicrobial treatment; increasing antimicrobial resistance may add to the difficulty or delay in administration of microbiologically effective therapy, leading to increased morbidity and mortality(Helms *et al*., 2012).

Salmonella evolved into a pathogen by acquiring pathogenicity determinants through horizontal gene transfer; similarly, it developed antibiotic resistance by virtue of mobile DNA elements (Lee *et al*., 2010). Mirza *et al*. (2010)were reported that antimicrobial resistance was transferable from *Salmonella* spp. to *Escherichia coli* as well as between other members of the intestinal normal flora. Plasmids are a major mechanism for the spread of antibiotic resistant genes in bacterial populations (Smalla *et al*., 2011).

3.4 Detection and Variation of CRISPR I and CRISPR II in *S enterica* (*S typhimurium* , *S entritidis* , *S typhi*)isolates

A total 18 of *S enterica* serotypes isolates from different clinical specimens (stool & blood) were subjected for detection CRISPR I & II of these 18(100%) & 13(72%) isolates were PCR – positive for CRISPR I & II respectively . The number of spacer of CRISPR I & II were between 5-13 &1-12 spacer respectively . The nucleotide length of direct repeat in CRISPR I & II was 29 as shown in table (3-4)

Table (3-4): CRSPR I and CRSPR II Identification results

Salmonella type	**Isolate**	**CRSPR1**					**CRSPR2**				
		PCR Product Length (bp)	**CRSPR Length**	**DR Length**	**Number of spacers**	**+ or -**	**PCR Product Length (bp)**	**CRSPR Length**	**DR Length**	**Number of spacers**	**+ or -**
Salmonella Typhi	S_ty29-1	763	333	29	5	+	1000	638	29	10	+
	S-ty30-1	778	333	29	5	+	-	-	-	-	-
	S-ty31-1	794	333	29	5	+	-	-	-	-	-
	S_ty32-1	808	333	29	5	+	-	-	-	-	-
	S_ty33-1	799	333	29	5	+	-	-	-	-	-
	S_ty34-1	796	333	29	5	+	-	-	-	-	-
Salmonella Typhimurium	S_TM1-1	1022	703	29	11	+	1094	89	29	1	+
	S_TM2-1	1020	702	29	11	+	1100	89	29	1	+
	S_TM3-1	1009	700	29	11	+	1298	638	29	10	+
	S_TM4-1	1004	821	29	13	+	2020	699	29	11	+
	S_TM26-1	1005	699	29	11	+	1456	150	29	2	+
	S_TM27-1	1004	698	29	11	+	1453	150	29	2	+
Salmonella Enteritidis	S_E6-1	1216	331	29	5	+	692	334	29	12	+
	S_E7-1	1442	676	29	10	+	1982	89	29	1	+
	S_E8-1	1440	758	29	12	+	1980	699	29	11	+
	S_E10-1	761	515	29	8	+	1433	89	29	1	+
	S_E11-1	758	455	29	7	+	1051	638	29	10	+
	S_E17-1	1233	639	29	10	+	1935	638	29	10	+

There is sufficient variation within CRISPR that can be used to discriminate between closely related *S enterica* a total of 18 of *S enterica* (*S enteritidis* , *S typhimurium* , *S typhi*) isolates from various source (stool and blood) were subjected for detection of CRISPR-I and CRISPR-II as show in figures (3-5) & (3-6).

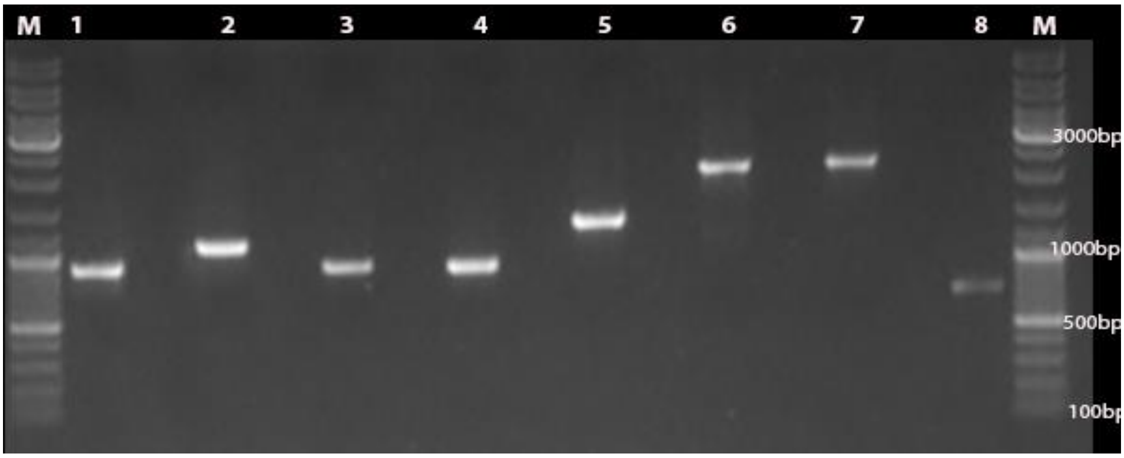


Fig. (3-5): Agarose gel electrophoresis at 75 volt (20mA) for 60 min for Crispr I, Lanes From left represent the following: 2- STM3, 1- STM4, 3- STM26, 4-STM27, 5- SE6, 6- SE7, 7- SE8, 8-SE10). Lane M, DNA ladder.

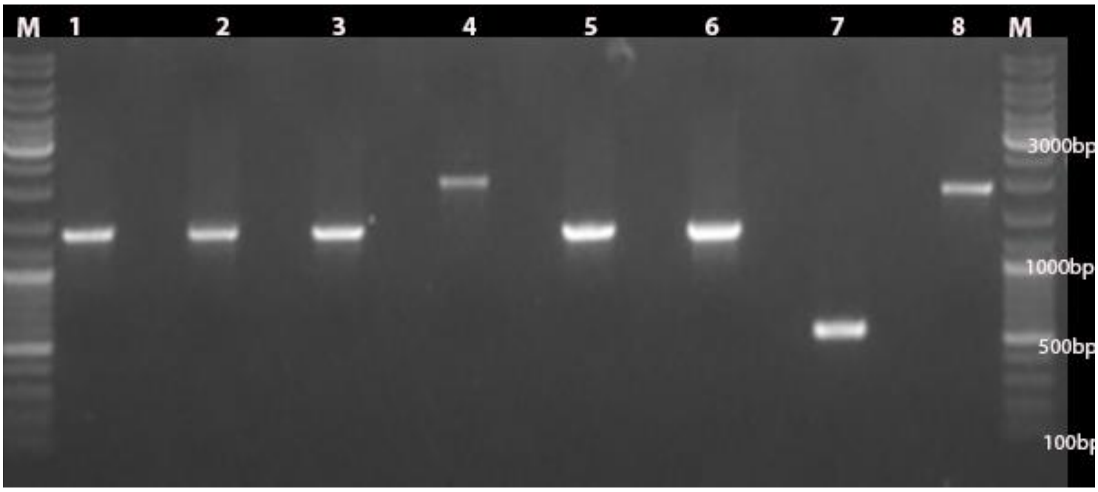


Fig. (3-6): Agarose gel electrophoresis at 75 volt (20mA) for 60 min for Crispr 2, Lanes From left represent the following: 1- STM1, 2- STM2, 3- STM3, 4- STM4, 5-STM26, 6- STM27, 7- SE6, 8-SE7). Lane M, DNA ladder.

The presence of CRISPR2 is frequently accompanied by the emergence of CRISPR1, CRISPRs were selected as a genotyping target because there were found to be one of the most rapidly evolving region in bacterial genomes the gene sequencing of *S enterica* isolates will help to evaluated the effectiveness of CRISPR typing for outbreak investigations and identify other potential genotyping targets.

Class 1 CRISPRCas systems are considered evolutionary ancestral systems. The class 2 systems have evolved from class 1 systems via
the insertion of transposable elements encoding various nucleases, and are now being used as tools for genome editing (Mohanraju *et al*., 2016).

To elevate the relationships between all detected CRISPR types of serotypes , a phylogenetic tree based on the spacer & DR sequence of the CRISPR types was constructed . The results showed that CRISPR types had a good correspondence with serotypes whereas strains belonging to the same CRISPR type were usually located on the neighboring branch as shown in figures (3-7, 3-8 , 3-9 & 3-10) .

Typical CRISPR arrays with direct repeat (DR) interspaced with spacer were predicted in isolates with a CRISPR system . DR of CRISPR-I and CRISPR-II isolates showing identical length 29 bp.

The direct repeat sequence alignment of CRISPR-I and CRISPR-II indicate the diversity of single nucleotide polymorphism found scattered in both CRISPR-I and CRISPR-II which can divided into 18 and 13 diverse patterns respectively clustering. The polymorphism of DR of CRISPR-I by phylogenetic tree construction indicated that all 18 patterns can divided into six linage (A-F) as show in figure (3-7) , whereas among 13 diverse patterns of CRISPR-II can classified into four linage (A-D) as show in figure (3-8). However, the sequence analysis indicate that the direct repeat region in both CRISPR-I and CRISPR-II has a high diversity.

Our results confirmed that it is feasible to conduct phylogenetic analysis and detect evolutionary distance based on the CRISPR loci of closely related species. However, this method only applies to bacteria with relatively conserved CRISPR system .

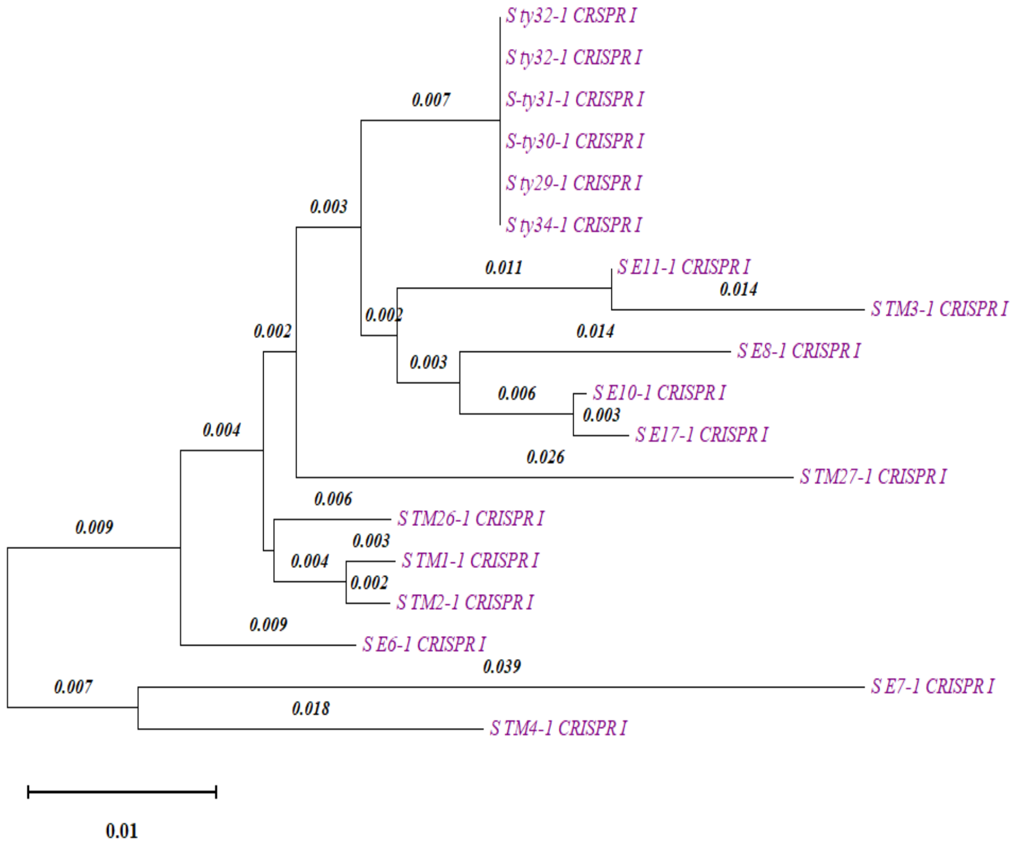


Fig (3-7) : CRISPR I Phylogenetic tree of studied *salmonella* isolates according to direct repeat sequences using Neighbor-joining method via MEGA-X Software.

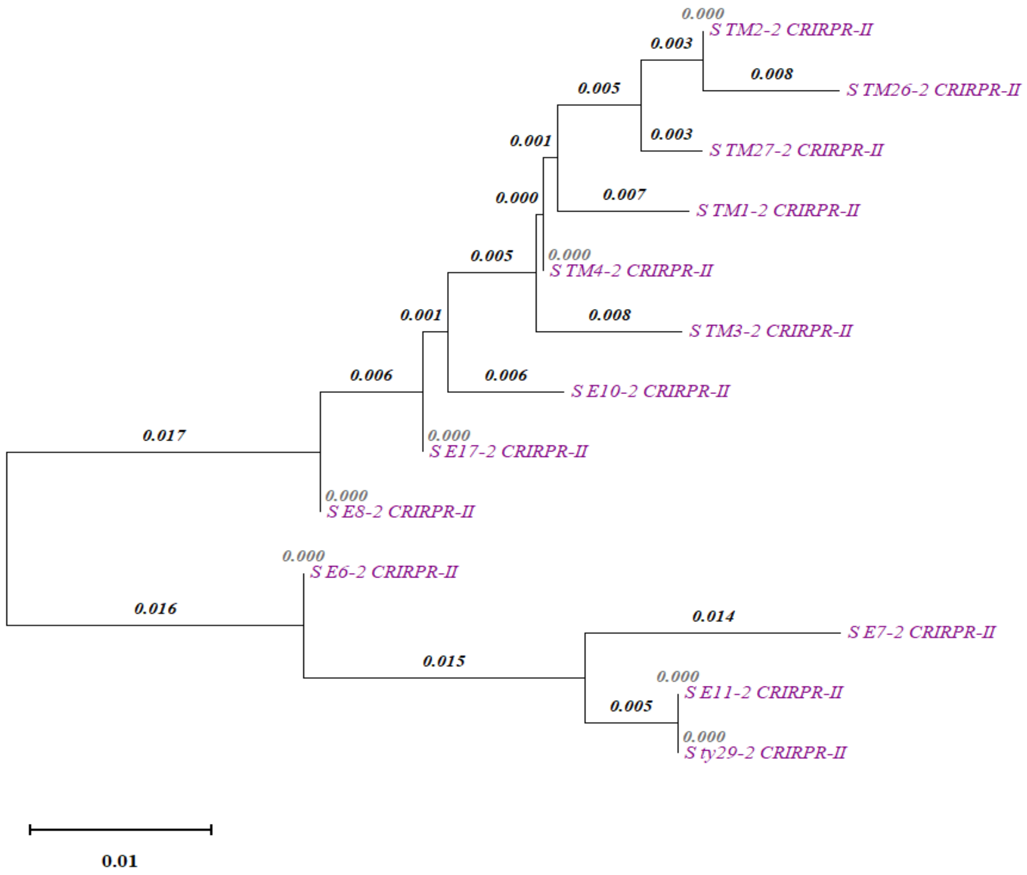


Fig (3-8) : CRISPR II Phylogenetic tree of studied *salmonella* isolates according to direct repeat sequences using Neighbor-joining method via MEGA-X Software.

From the genetic similarity value of *S enterica* serotype according to repeat sequence for CRISPR-I and CRISPR-II was shown in table (3-5) & (3-6), it was found in CRISPR-I there is maximum similarity between serotype such as SE11 & STM3 at (100%) and minimum similarity STM4 & STY29 at (40%). While in CRISPR-II , the maximum similarity between serotype SE11 & STY29 at (100%) and the minimum similarity matrix between serotype SE7 & STM4 at (19%) , the result revealed high percentage of genetic variation among studied isolates and introduced great genetic differentiation of all used isolates.

Table (3-5) : CRISPR I Similarity in percent of studied *salmonella* isolates according to direct repeat sequences.

	SE 6	SE 7	SE 8	SE 10	SE 11	SE 17	ST M1	ST M2	ST M3	ST M4	ST M26	ST M27	Sty 29	Sty 30	Sty 31	Sty 32	Sty 33	Sty 34
SE6	100%	79%	89%	67%	61%	83%	90%	90%	86%	95%	90%	88%	45%	45%	45%	45%	45%	45%
SE 7	93%	100%	92%	67%	69%	92%	93%	93%	91%	94%	93%	91%	50%	50%	50%	50%	50%	50%
SE 8	89%	78%	100%	68%	61%	82%	89%	89%	87%	91%	89%	87%	45%	45%	45%	45%	45%	45%
SE 10	97%	82%	98%	100%	78%	89%	98%	97%	97%	95%	98%	95%	65%	65%	65%	65%	65%	65%
SE 11	100%	95%	99%	88%	100%	100%	100%	100%	100%	97%	100%	97%	73%	73%	73%	73%	73%	73%
SE 17	98%	92%	97%	72%	72%	100%	98%	98%	94%	95%	98%	95%	53%	53%	53%	53%	53%	53%
ST M1	97%	86%	97%	73%	67%	90%	100%	99%	94%	95%	98%	96%	49%	49%	49%	49%	49%	49%
ST M2	97%	86%	96%	73%	67%	90%	99%	100%	95%	95%	99%	96%	49%	49%	49%	49%	49%	49%
ST M3	94%	83%	94%	73%	67%	86%	94%	95%	100%	91%	95%	92%	49%	49%	49%	49%	49%	49%
ST M4	88%	74%	85%	61%	55%	75%	82%	81%	78%	100%	81%	80%	40%	40%	40%	40%	40%	40%
ST M26	97%	86%	97%	73%	67%	90%	98%	99%	95%	95%	100%	96%	49%	49%	49%	49%	49%	49%
ST M27	95%	84%	95%	72%	64%	87%	96%	96%	92%	93%	96%	100%	48%	48%	48%	48%	48%	48%
Sty 29	97%	91%	97%	98%	98%	97%	98%	98%	98%	94%	98%	96%	100%	100%	100%	100%	100%	100%
Sty 30	97%	91%	97%	98%	98%	97%	98%	98%	98%	94%	98%	96%	100%	100%	100%	100%	100%	100%
Sty 31	97%	91%	97%	98%	98%	97%	98%	98%	98%	94%	98%	96%	100%	100%	100%	100%	100%	100%
Sty 32	97%	91%	97%	98%	98%	97%	98%	98%	98%	94%	98%	96%	100%	100%	100%	100%	100%	100%
Sty 33	97%	91%	97%	98%	98%	97%	98%	98%	98%	94%	98%	96%	100%	100%	100%	100%	100%	100%
Sty 34	97%	91%	97%	98%	98%	97%	98%	98%	98%	94%	98%	96%	100%	100%	100%	100%	100%	100%

Table (3-6) : CRISPR II Similarity in percent of studied *salmonella* isolates according to direct repeat sequences.

	SE6	SE7	SE8	SE10	SE11	SE17	STM1	STM2	STM3	STM4	STM26	STM27	Sty29
SE6	100%	99%	97%	97%	99%	97%	97%	97%	97%	97%	97%	97%	99%
SE7	19%	100%	19%	28%	35%	19%	24%	25%	32%	19%	35%	30%	35%
SE8	48%	48%	100%	99%	48%	91%	99%	99%	99%	99%	99%	99%	48%
SE10	34%	50%	70%	100%	50%	64%	92%	93%	98%	70%	98%	98%	50%
SE11-2	54%	98%	53%	77%	100%	53%	68%	71%	90%	53%	87%	82%	100%
SE17	53%	53%	99%	99%	53%	100%	99%	99%	99%	99%	99%	99%	53%
STM1	36%	47%	74%	97%	47%	68%	100%	98%	98%	75%	98%	98%	47%
STM2	36%	49%	74%	99%	48%	68%	98%	100%	100%	75%	100%	100%	48%
STM3	30%	53%	62%	88%	52%	57%	83%	84%	100%	63%	96%	93%	52%
STM4	48%	48%	99%	100%	48%	91%	100%	100%	100%	100%	100%	100%	48%
STM26	26%	50%	54%	76%	43%	50%	72%	73%	83%	54%	100%	81%	43%

STM27	**32%**	**51%**	**66%**	**92%**	**50%**	**61%**	**88%**	**89%**	**98%**	**66%**	**99%**	**100%**	**50%**
Sty29	**54%**	**98%**	**53%**	**77%**	**100%**	**53%**	**68%**	**71%**	**90%**	**53%**	**87%**	**82%**	**100%**

The CRISPR assay found in *S. enterica* varied in length and spacer content, in this study the length of different CRISPR arrays varied greatly with the number of spacer ranging from 1 to 13 , the longest CRISPR locus contained 13 unique spacer with most isolates contained 5 or 11 spacer. The number of spacer for CRISPR-I was between 5-13 spacer and CRISPR-II was between 1-12 spacer.

The number of spacer in CRISPR-I and CRISPR-II were different. Our study found that CRISPR-I has diverse spacer in term number and nucleotide variation more than CRISPR-II.

The alignment comparison of CRISPR-I and CRISPR-II spacer sequences was performed in *S enterica* .The phylogenetic tree constructed from the sequence alignment of CRISPR-I spacer show that each serotype was divided in group was shown in figure (3-9). In contrast to CRISPR-I, the phylogenetic tree constructed from sequence alignment of the CRISPR-II spacer was unable to discriminate between *S enterica* serovars was shown in figure (3-10) .

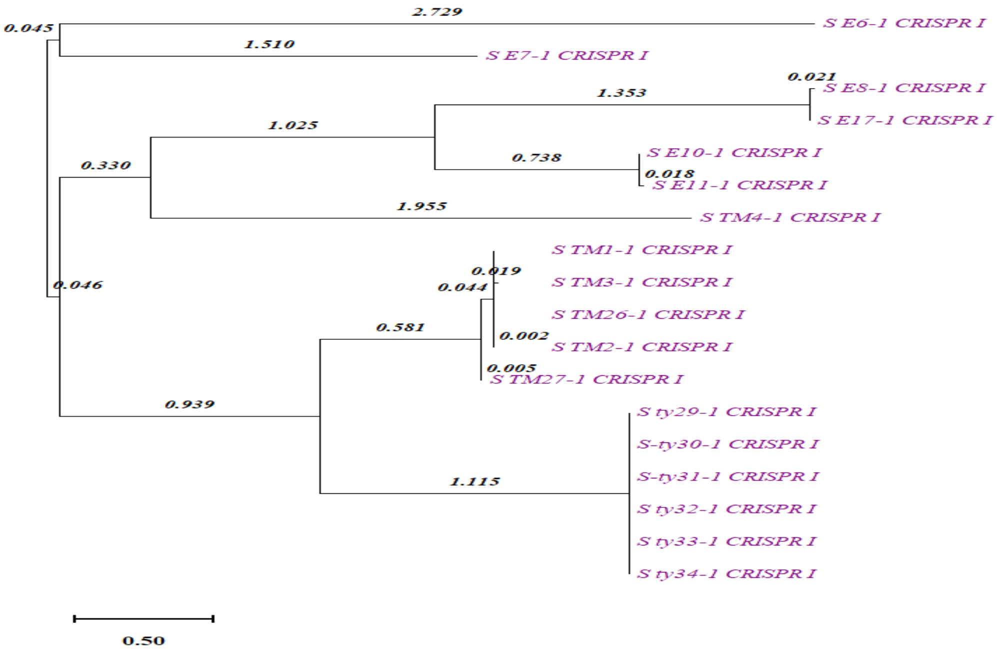


Fig (3-9) : CRISPR I Phylogenetic tree of studied *salmonella* isolates according to Spacer sequences using Neighbor-joining method via MEGA-X Software.

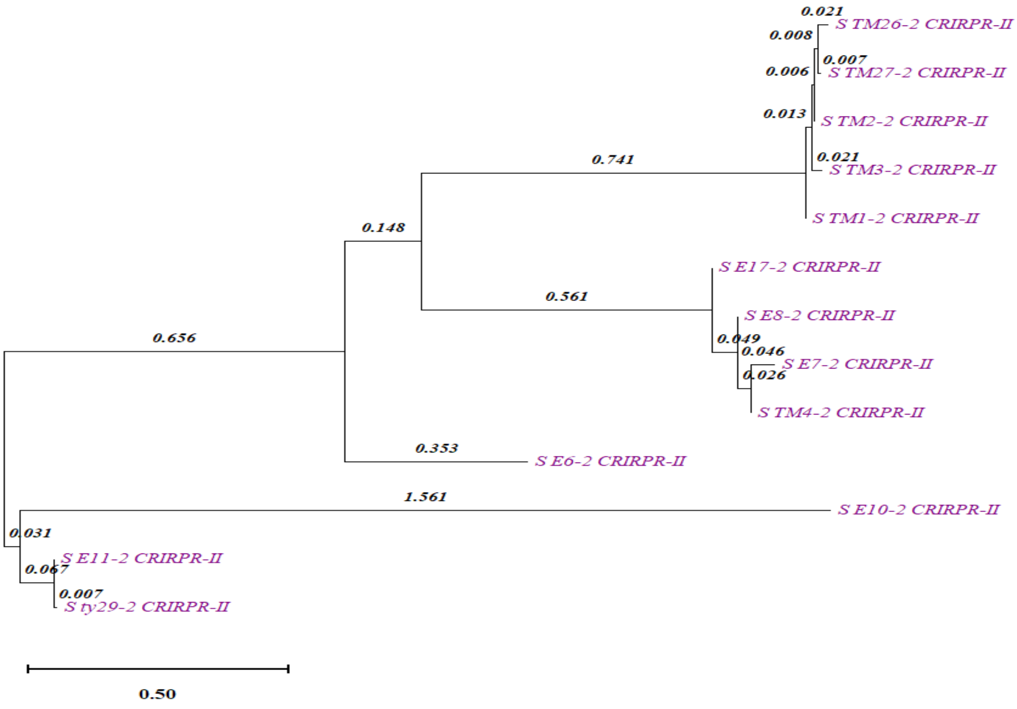


Fig (3-10) : CRISPR II Phylogenetic tree of studied *salmonella* isolates according to Spacer sequences using Neighbor-joining method via MEGA-X Software.

The genetic similarity value of *Salmonella enterica* serotype according to spacer sequence for CRISPR-I and CRISPR-II was shown in tables (3-7) & (3-8), it was found that in CRISPR-I there is a maximum similarity between Salmonella serotypes STM26 & STM1 at (100%) and minimum similarity between SE6 & Sty29 at (11%) ,while in CRISPR-II the maximum similarity between serotypes STM4 & SE7, STM4 &SE8 , STY29 &SE11 at (100%) and the minimum similarity between serotypes STM26 & SE6 at (10%) cluster analysis based on combined similarity matrix grouped all isolates in different clusters indicating a genetically variability of isolates.

Table (3-7) : CRISPR I Similarity in percent of studied *salmonella* isolates according to spacer sequences.

	SE6	SE7	SE8	SE10	SE11	SE17	STM1	STM2	STM3	STM4	STM26	STM27	Sty29	Sty30	Sty31	Sty32	Sty33	Sty34
SE6	100%	28%	27%	21%	19%	24%	31%	31%	29%	25%	30%	28%	11%	11%	11%	11%	11%	11%
SE7	31%	100%	28%	23%	20%	27%	31%	31%	31%	31%	31%	31%	18%	18%	18%	18%	18%	18%
SE8	27%	27%	100%	30%	26%	83%	31%	31%	32%	24%	31%	32%	12%	12%	12%	12%	12%	12%
SE10	32%	32%	45%	100%	88%	45%	38%	38%	37%	35%	38%	37%	14%	14%	14%	14%	14%	14%
SE11	32%	31%	45%	100%	100%	45%	38%	38%	37%	35%	38%	38%	16%	16%	16%	16%	16%	16%
SE17	28%	30%	99%	36%	31%	100%	35%	35%	36%	26%	35%	36%	15%	15%	15%	15%	15%	15%
STM1	33%	31%	34%	27%	24%	32%	100%	99%	97%	31%	99%	94%	24%	24%	24%	24%	24%	24%
STM2	33%	31%	34%	27%	24%	32%	100%	100%	97%	31%	99%	94%	24%	24%	24%	24%	24%	24%
STM3	32%	31%	35%	27%	23%	33%	98%	98%	100%	30%	98%	93%	24%	24%	24%	24%	24%	24%
STM4	23%	27%	22%	21%	19%	20%	26%	26%	25%	100%	26%	26%	13%	13%	13%	13%	13%	13%
STM26	33%	31%	34%	27%	24%	32%	100%	100%	98%	31%	100%	95%	24%	24%	24%	24%	24%	24%
STM27	31%	31%	35%	27%	24%	32%	95%	95%	94%	31%	95%	100%	24%	24%	24%	24%	24%	24%
Sty29	26%	39%	29%	22%	22%	29%	54%	54%	53%	33%	54%	53%	100%	100%	100%	100%	100%	100%
Sty30	26%	39%	29%	22%	22%	29%	54%	54%	53%	33%	54%	53%	100%	100%	100%	100%	100%	100%
Sty31	26%	39%	29%	22%	22%	29%	54%	54%	53%	33%	54%	53%	100%	100%	100%	100%	100%	100%
Sty32	26%	39%	29%	22%	22%	29%	54%	54%	53%	33%	54%	53%	100%	100%	100%	100%	100%	100%
Sty33	26%	39%	29%	22%	22%	29%	54%	54%	53%	33%	54%	53%	100%	100%	100%	100%	100%	100%

Sty 34	**26%**	**39%**	**29%**	**22%**	**22%**	**29%**	**54%**	**54%**	**53%**	**33%**	**54%**	**53%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**

Table (3-8) : CRISPR II Similarity in percent of studied *salmonella* isolates according to spacer sequences.

	SE6	SE7	SE8	SE10	SE11	SE17	STM 1	STM 2	STM 3	STM 4	STM26	STM27	Sty29
SE6	100%	41%	41%	38%	36%	41%	43%	43%	43%	41%	43%	43%	36%
SE7	7%	100%	37%	13%	13%	33%	19%	19%	19%	37%	19%	19%	13%
SE8	18%	100%	100%	31%	37%	91%	43%	43%	43%	100%	43%	43%	37%
SE10	12%	25%	21%	100%	24%	20%	26%	26%	28%	21%	27%	27%	24%
SE11	18%	40%	40%	38%	100%	40%	36%	36%	36%	40%	36%	36%	100%
SE17	20%	100%	100%	32%	40%	100%	44%	44%	44%	100%	44%	44%	39%
STM 1	14%	38%	31%	28%	24%	29%	100%	100%	100%	31%	100%	100%	24%
STM 2	14%	38%	31%	28%	24%	29%	100%	100%	100%	31%	100%	100%	24%
STM 3	12%	31%	26%	24%	20%	24%	82%	82%	100%	26%	93%	90%	20%
STM 4	18%	100%	100%	31%	37%	91%	43%	43%	43%	100%	43%	43%	37%
STM 26	10%	27%	22%	21%	17%	21%	71%	71%	80%	22%	100%	78%	17%
STM 27	13%	34%	28%	26%	22%	26%	88%	88%	97%	28%	97%	100%	22%
Sty 29	18%	40%	40%	38%	100%	39%	36%	36%	36%	40%	36%	36%	100%

CRISPR typing was performed by combining CRISPR I & II .The resulting profile for each strain after the spacer sequence was arrayed in conventional CRISPR typing & the strains were found to have high genetic polymorphism.

However , the CRISPR shows a high diversity of spacer within those *S enterica* serovar isolates which were sampled from one geographical location. Since CRISPR spacer which are undergoing rapid horizontal gene transfer events, can change rapidly within a few generation of *S enterica* growth in a host .

The spacer was conserved in CRISPR-I and absent in 5 isolates in CRISPR-II , the variability of CRISPR-I & II spacer either resulted from the duplication of single spacer and /or deletion of single spacer.

The CRISPR regions could be used as an original target for the development of PCR assays specific for particular *Salmonella* serotypes containing constant and unique spacer. The repetitive distribution of the spacer might related to the sequence recombination of the repeat , & the recombination process might be accompanied by an increase & deletion of the spacer as well as the position change of the spacer .

The DR sequence were different & part of the spacer was derived from foreign gene . The under antibiotic pressure , the bacterial CRISPR sequence may mutate , allowing the bacteria to acquire exogenous resistant gene & survive the variation in CRISPR sequence & the diversity of spacer sequence might be related to the resistance of *Salmonella* . So the variety of spacers and direct repeat (DR) sequence could be applied into subtyping or tracing strains from different sources.

The spacer sequences vary in size from 26-72 base pairs. The spacers have similar lengths within a particular CRISPR array, but two identical spacers are not found in the same array (Lillestøl *et al*., 2006). Although it is believed that the spacer sequences have originated from foreign mobile genetic elements, just a small portion of all known spacer maps have been found to contain extra chromosomal sequences from phages and plasmids (Shah *et al*., 2009). CRISPR analysis studies have shown a significant polymorphism in the number and type of spacer sequences within different strains of a particular species, thereby turning them into a suitable tool for epidemiological studies. Besides, there is an offer that CRISPRs can impact the autoimmunity by use of spacers that target self-genes so CRISPRs can incur an autoimmune fitness cost by incorporation of nucleic acids, that may justify the abundance of degraded CRISPR systems across prokaryote (Stern *et al*., 2010).

Diversity in spacer number and nucleotide sequences have been report even in the same bacterial species. This probably due to the differences between the habitat, hosts, as well as loss or duplication of the acquired CRISPR arrays, which resulted in very extensive diversity that occur both inter-species and intra-species (Fabre *et al*., 2012;Sheludchenko *et al*., 2015; Koonin & Makarova, 2019). Moreover, these variation arrays are able to be transferred from the parent strain to the daughter strain via vertical transfer (Iranzo *et al*., 2013).

Some bacterial strains may have spacer sequences with different numbers at the same CRISPR loci, offering a strain-specific polymorphism that can be used in phylogeny and epidemiological studies (Beuvink *et al*., 2007). But, it is noteworthy that strain-specific polymorphism that can be used in phylogeny and epidemiological studies (Beuvink et al., 2007). However, some bacterial species (about 50%) did not acquire CRISPR loci in their genomes, and also some bacterial species acquired spacers at a higher rate, they could not be sub-grouped through the CRISPR based- molecular subtyping (Subtyping, 2014). Spacer-oligonucleotide typing or “Spoligotyping” was the first use of spacer information for bacterial subtyping applications (Kamerbeek *et al*., 1997). The principle is the PCR amplification of the CRISPR arrays with labeled primers that detect applications (Kamerbeek *et al*., 1997).

Simon Le Hello and coworkers in 2013 reported that CRISPR sequences are very applicable targets for subtyping of *Salmonella enteritidis* isolates. They concluded that since CRISPR spacer content can be easily obtained from short-read DNA sequences, it could be used to identify particular *Salmonella* and probably other bacterial pathogens (Zheng *et al*., 2014; Le Hello *et al*., 2015)

CRISPR arrays contain a series of sequence-specific conserved repeats flanking unique inserts known as spacers. New spacers are derived from invading mobile genetic elements and integrated into CRISPR arrays through the activity of Cas proteins (Barrangou *et al*., 2007). Each spacer encodes the targeting sequence of a given CRISPR array-derived small crRNA (Wiedenheft *et al*., 2011). The spacer sequences of a given bacterial strain therefore constitute a history of past CRISPR-Cas-mediated interactions. Studies in *Salmonella*, *Campylobacter*, *Mycobacterium,* and *Corynebacterium* isolates have shown that comparing the spacer sequences of a given strain can provide a phylogenetic bar code to rapidly track pathogenic strains during outbreaks in a process termed CRISPR typing (Fabre *et al*., 2012).

As unique CRISPR spacer content primarily represents independently acquired and evolutionarily selected short ribonucleoprotein targeting sequences with low genomic background complementarity (Stern *et al*., 2010), their sequences can be used to highlight previously encountered genetic elements within complex genomic data.

As the CRISPR-Cas system is composed of relatively conservative cas genes and high variable CRISPR loci, which are repeated sequences intercalated with different short “spacer” sequences matching to foreign DNA elements such as phage or plasmid genomes (Goldberg *et al*., 2014).

However, it has not been reported whether acquisition of new spacers and loss of old spacers in response to phage and/or plasmids has occurred in *S. enterica*. In addition, the function of CRISPR-Cas systems in Salmonella has not been revealed in previous studies. However, the previous study showed CRISPR spacers are closely related to the serotypes of *Salmonella*, which is to say, a specific *Salmonella enterica* serovar has its unique spacers or spacer arrangements, and some unique serotypes have complicated spacers (Li *et al*., 2017).

Spacers that are identical to known sequences provide useful information because similarities between spacer and proto-spacer sequences can provide some suggestion of the probable origin ofthe spacers (Horvath *et al*., 2008). We therefore performed homology analysis to detect the targets of CRISPR. It should be noted that the study by (Dy *et al*., 2013) were did not completely determine whether the potential targets identified in our search are true spacer origins or would support CRISPR interference function, as most of the homologous regions are part of the spacer sequence or outside of seed regions. Even so, we cannot rule out the relationship between spacers and potential targets because the mutations may occur in proto-spacer regions after the acquisition of CRISPR spacers.

The presence of a spacer into the CRISPR array promotes the acquisition of various spacers in a second re infection from the previously encountered invader: this biological process is termed primed adaptation (Datsenko *et al*., 2012; Swarts *et al*., 2012).

3.4.1 In silico PCR-RFLP:-

The nucleotide sequence analysis of the CRISPR-I & II of *S. enteric* (*S enteritidis, S typhimurium , S typhi*) revealed a high diversity in the number & nucleotide of the direct repeat region as well as the nucleotide sequence of the spacer region . The results in silico PCR-RFLP using BstUI restriction enzyme & simulation separation in gel electrophoresis was demonstrated that the enzyme was able to generate several DNA banding pattern with different size .these different patterns correspond to *S. enterica* (*S enteritidis, S typhimurium , S typhi*) isolated from different specimen (stool & blood) . The obtained fragments ranged from 2 – 330 bp in CRISPRI , while fragments ranged from 3- 375 bp in CRIAPRII as shown in figures (3-11 , 3-12) and **appendix (3)**

Some of *S. typhimurium* isolates showed closely related DNA banding patterns indicated that it may be originated from the same linage as show in figure (3-12) such as STM27 isolates closely related in STM26 in CRISPRII .

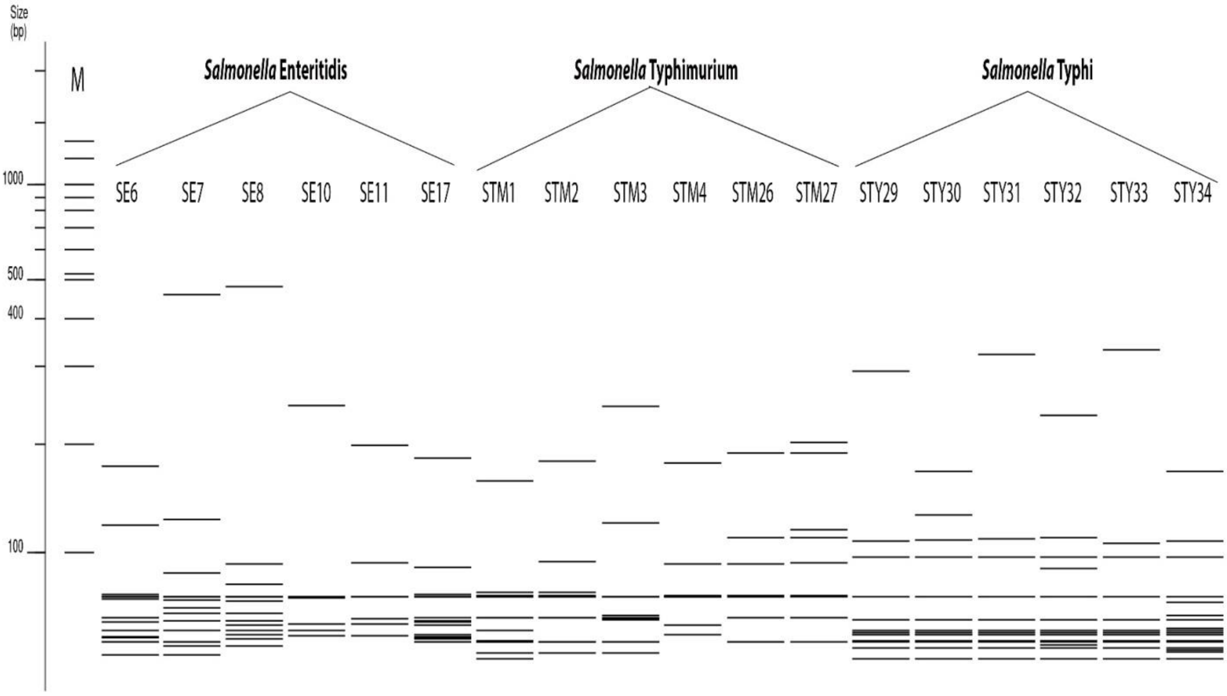


Fig (3-11): In silico PCR-RFLP of CRSPR I of studied *salmonella* isolates, digested by *BST*UI and simulation separation in Agarose Gel Electrophoresis.

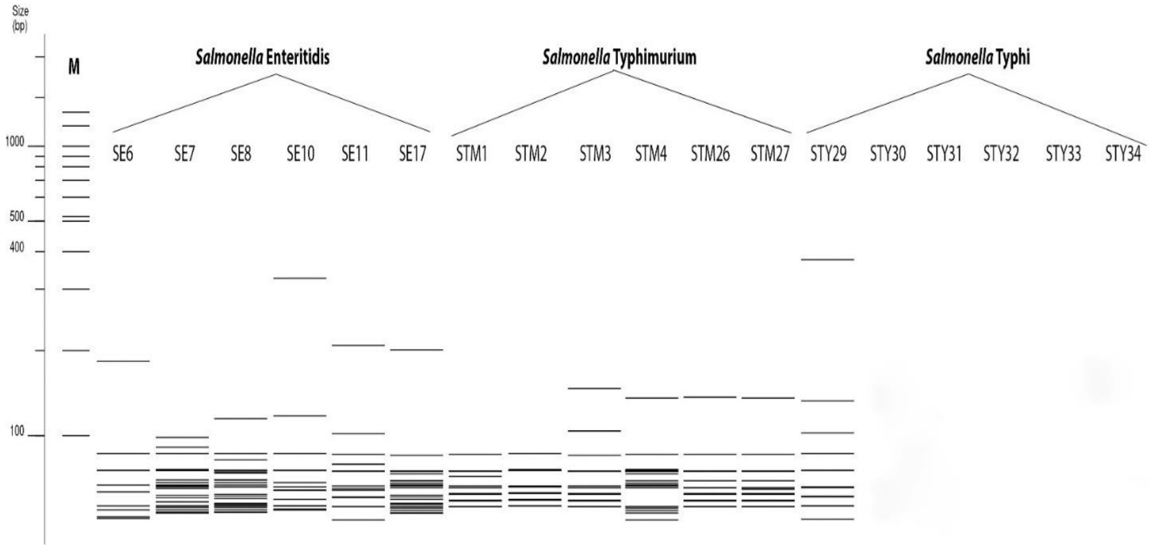


Fig (3-12): In silico PCR-RFLP of CRSPR II of studied *salmonella* isolates, digested by *BST*UI and simulation separation in Agarose Gel Electrophoresis.

Based on the finding, CRISPR array of *S. enterica* are divers allowing them to be used as an alternative tool for tracking the *S. enterica* outbreak.

Although this study was report an in silico simulation methods it is indicates that the nucleotide sequence and thus the results can be reliable & feasible .

CRISPR sequences are widely distributed in bacterial genomes & composed by direct repeats & spacers . since spacers show chronological acquisition and a high degree of polymorphism . Studies using CRISPR to determine phylogenetic relationships of closely related taxa , source tracking &geographical origin of different *S.enterica* spp. have also been performed .

CRISPR typing is a method used to reveal the genetic relationship of bacterial isolates from different resources & genetic relationship among different strains also it reveal the phylogenetic difference among isolates belong to the identical serotype and also a good correspondce with *Salmonella* serotype . The CRISPR region varies between various organisms according to the number and also the size (from 23-55 base pairs in length). These repeats that are clustered into one or more loci on the chromosome are often partially palindromic with the ability to form hairpin structures. On average, the bacterial genome contains three CRISPR arrays, compared to five CRISPR arrays found in the archaeal genome (Grissa *et al*., 2007). A considerable characteristic of the CRISPR arrays is the ability of their transcripts to form RNA secondary structures (Kunin *et al*., 2007).

3.5 Whole genome sequencing

The significant use of WGS technology has clearly been demonstrated in epidemiological studies and outbreak detection for Salmonella and other enteric pathogens revealing outbreak associations missed by standard Pulsed-Field Gel Electrophoresis (Henk, 2014; Scaltriti *et al*., 2015)

At a genomic level, this study tried to discriminate between closely related strains, *Salmonella enterica* subsp. enterica serovar Typhimurium (STY9) and *Salmonella enterica* subsp. enterica serovar Typhi (SalT33) isolates , by using whole genome sequencing depending on the next-generation sequencing method. According this technique, two strains of *Salmonella enterica* (STY9,SalT33) were subjected to whole genome sequencing. The resulted data were analyzed to calculate the General characteristics of *Salmonella enterica* genomes, Comparative genome analysis, Gen Annotation, Mapping and Variants calling.

3.5.1 General characteristics of *Salmonella enterica* genomes

In the present study, whole-genome sequencing method generated a raw paired-end reads. For each genome, total number of bases sequenced, total number of nucleotides, GC content (%),Genome Size and Contigs. Regarding the first raw data in *Salmonella enterica* subsp. enterica serovar Typhimurium (STY9), A 3.4G bp of total nucleotides bases were produced and total sequences were 12,097,554 bp. The GC content was 52.2% . In *Salmonella enterica* subsp. enterica serovar Typhi (SalT33), A 2.1 G bp of total nucleotides bases were produced and total sequences were 6,998,132 bp. The GC content was 52.12% .

To reduce bias in the data analysis, read trimming and filtering was performed using specific bioinformatics tools. After filtering, the same statistics criteria were calculated again. The analysis of the filtered raw sequences for (STY9) generated 3.3 G bp of total nucleotides bases, 11,179,659 total sequences , 52.2% GC . Regarding (SalT33) the results of data analysis were 2.0 G bp of total nucleotides bases , 6,708,477 total sequences , 52.12% GC. After assembling, the final draft genome size of was 4,687,295 bp for *Salmonella enterica* subsp. enterica serovar Typhimurium (STY9) and 4,679,911 bp for *Salmonella enterica* subsp. enterica serovar Typhi (SalT33) . Additionally, the draft genomes of (STY9) and (SalT33) contained on 122 and 62 contigs. General characteristics of *Salmonella enterica* genomes are summarized in Table (3-9).

Table (3-9): General characteristics of Salmonella enterica subsp. enterica serovar Typhimurium STY9 genome and Salmonella enterica subsp. enterica serovar Typhi SalT33 after filtration and assembly

Characteristics	**STY9**	**SalT33**
Total sequences	11,179,659 bp	6,708,477 bp
Total nucleotides	3,365,130,082 bp	2,030,160,572 bp
GC%	52.2	52.12
Genome Size	4,687,295 bp	4,679,911 bp
Contigs	122	62

Regarding genome size, the results of this study noted very slightly differences among *Salmonella enterica* genomes, where the genome of (STY9) serovars was larger than (SalT33). A study by (Baddam *et al.*, 2012) appeared that the genome size of *Salmonella typhi* was approximately 4,744,056 with GC content of 53.21% . The variation in the sie genome among *S. enterica* sp. isolates caused by extremely genomic rearrangements such as duplication and indels.

The complete genome of S. Typhi, Ty2, was reported to have been isolated from Russia in the early 1970s (Wan Makhtar *et al.*, 2021). The Ty2 strain is the basis for the production of vaccines, and is the parent of the Ty21a and CVD908 mutant strains whose descendants were used in live attenuated vaccine trials (Deng *et al.*, 2003). The size of the circular chromosomes of Ty2 and CT18 are also slightly different, of which Ty2 has 4,791,961 bp with an average G+C content of 52.05%, while CT18 has a total circular chromosome size with 4,809,037 bp with G+C content of 52.09% (Wan Makhtar *et al.*, 2021). In its population, *S. Typhi* also revealed a richness in genome composition. *S. Typhi* was observed to have a higher level of chromosome rearrangement in wild-type strains relative to *S. Typhimurium*. Due to the recombination between The ribosomal RNA (rrn) operons, which are deletions, duplications, translocations, and inversions, the chromosome rearrangements could be formed.

3.5.2 Comparative genome analysis

The whole genomes of the studied *S. enterica* isolates (STY9, SalT33) were compared with each other or with the reference genome (NC_003197.2 , NC_003198.1) respectively to identify genomic rearrangements, conserved genomic regions and segmental gain or loss. This type of analysis is an attempt to understand how these genomes evolved and what are the main differences between them. At the whole-genome level, two patterns of Comparative genome analysis were performed, Multiple genome alignment comparison and BLAST Ring Image Generator (BRIG).

3.5.2.1Multiple genome alignment comparison of *Salmonella enterica genomes*

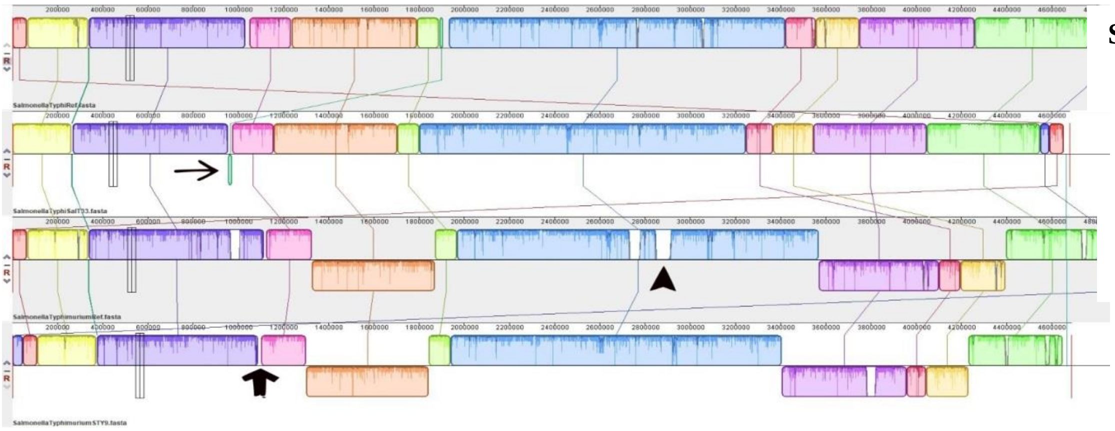
In this study, Multiple genome alignment comparison among the studied genomes of *S. enterica* and the reference genome was performed by using progressive Mauve. This type of comparative analysis identifies evolutionary events by aligning homologous regions of sequence resulting conserved segments, called locally collinear blocks (LCBs). Each LCB represents a homologous region of sequence conserved among all the genomes being aligned (Darling *et al.,* 2004).

However, the results of this study revealed 13 LCBs among the aligned genomes (Reference genome and the studied genomes of *S. enterica* (SalT33) with different lengths . On the other hand , this study revealed 12 LCBs among alignment genome (Reference genome and the studied genome of *S. enterica* (STY9) with different lengths . The lengths of LCBs 3 in *S. typhi* was 8 kbp . The presence of this region only in the studied strains may indicates to a pattern of segmental gain during the course of evolution. Moreover, the results of this study showed different patterns of genome rearrangement. In more details, there are several regions lack detectable sequence homology among the studied genomes. These regions represent sequences outside colored LCBs that visualized (marked as a thick arrow) in figure (3-13). All the studied genomes of *S. enterica sp.* possessed regions with no sequence homology and the large region noted on *S. typhimurium* . The presence of this regions on the studied genomes may be related to frequent nucleotide substitutions that occurred by mutations.

In addition, the results of this study determined several sequence regions inside each LCB that were not aligned with other genome. These regions considered as a specific sequence element to a particular genome not shared to other genomes, these regions marked as arrow head in figure (3-13). Accordingly, these regions were noted in *S. typhimurium* more than *S. typhi*. The presence of these genetic segments refers to several evolutionally changes such as segmental insertion or deletion (indels) that could be related to the horizontal gene transfer. In addition, the genome of *S. typhi* revealed one reciprocal LCBs, as illustrated on figure (3-13) with thin arrow. These blocks are regions aligned in the reverse complement (inverse) orientation suggesting pattern of genomic rearrangements in *S. typhi* (SalT33) genome.

However , all the studied genomes of *S. enterica* (STY9, SalT33) showed different patterns of genomic rearrangements with each other or with the reference genome. Similarly, these genomes revealed conserved regions indicating the shared ancestry with the reference genome. In spite of this study may be the first study in Iraq that compared between *S. enterica* strains (STY9, SalT33)at whole genome level.

Globally, the evolution of epidemic strains of *S. enterica* has been significantly influenced by large-scale evolutionary events such as horizontal gene transfer, lateral gene transfer and genomic rearrangement such as inversions or relocations of DNA fragments, where observation of genome rearrangements between both closely related and divergent organisms represents a major force understanding evolution of *S. enterica*. For that reason, identification of genome rearrangements among *S. enterica* strains of Babylon Province provided further insights into their history of evolution.



SalT33 reference genome

STY9 reference genome

Fig. (3- 13): Comparative genome alignment of STY9 and SalT33 samples in comparison to reference genomes (NC_003197.2 , NC_003198.1). Sequence homology among genome contigs was assigned for each LCB with a unique color. Colored lines used to connect between similarly colored LCBs. Genomes from up to down are: reference genome of *Salmonella Typhi*, *Salmonella Typhi SalT33*, reference genome of *Salmonella Typhimurium* and *Salmonella Typhimurium STY9*. Symbols represent the following events: arrow head= sequence elements specific to a particular genome; thin arrow=reciprocal LCBs; thick arrow=Sequences outside colored LCBs that do not have sequence homologs in the other genomes.

3.5.2.2 Identification of sequence similarity among *Salmonella enterica* genomes for chromosome.

After analysis the whole genome of the studied *S. enterica* strains (typhimurium and typhi), this study identified the sequence similarity at the chromosomal Level.

Moreover, genome comparisons among the studied strains were performed to identify the sequence similarity. In comparison with the reference genome, the results of this study revealed that the sequence similarity in the SalT33 strain was higher than the STY9 strain , where STY9 have more gaps than SalT33 for all studied *S. enterica* strains (typhimurium and typhi). These gaps represent a missing genes or gen clusters that in turn suggesting that STY9 underwent to large scale of evolutionary events such as segmental gain or loss more than SalT33. Accordingly, the high rates of gaps in STY9 strain indicated that *S. enterica* typhimurium subjected to higher genome changes than SalT33. In addition, the results of this study was revealed that the sequence similarity to the reference genome was higher in SalT33 strain than STY9 strain indicating to lower genome changes in SalT33 strain than other studied strains. The results of multiple genome comparisons among STY9 and SalT33were visualized as circular plot and shown in figures (3-14, 3-15).

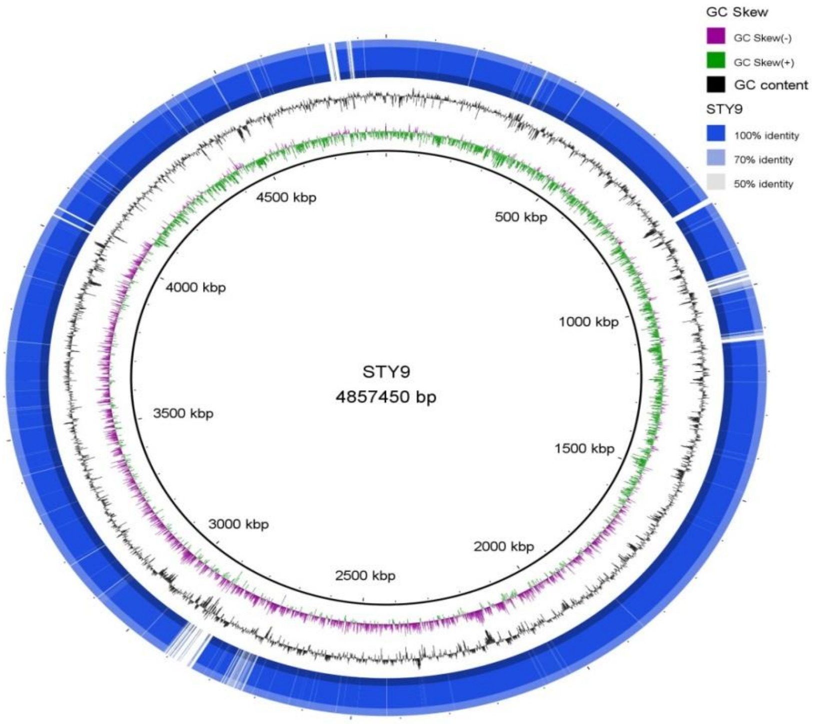


Fig (3-14): Comparative genome analysis against Reference sequence the structural differences in genome of STY9.

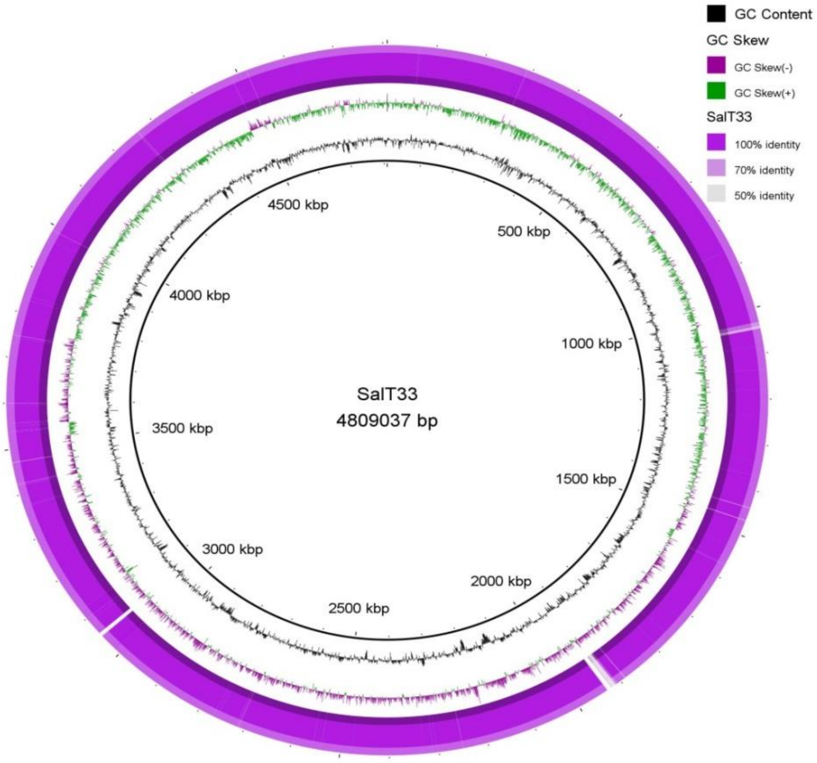


Fig (3-15): Comparative genome analysis against Reference sequence the structural differences in genome of SalT33

3.5.3 Annotation

In the present study, the whole genome sequences of the studied *Salmonella enterica* subsp. enterica serovar Typhimurium (STY9) and Salmonella enterica subsp. enterica serovar Typhi (SalT33) were annotated to identify the coding regions (genes) in a genome, the locations of genes and its function. In addition, comparative proteome analysis was performed for each genome. According to genome annotation, the results of this study included General characteristics of genome annotation, proteome comparison, General predicted subsystem groups and identification of the specialty genes (Antibiotic resistance, Drug target, Transporter and Virulence factors genes).

3.5.3.1 General characteristics of genome annotation for *Salmonella enterica* strains (STY9 , SalT33).

This study resulted several features regarding genome annotation of *S. enterica* strains (STY9 , SalT33). These features included the number of protein coding sequences (CDS), transfer RNA (tRNA) genes, and ribosomal RNA (rRNA) genes. Moreover, the function of these proteins was predicted for each genome. Accordingly, the results of **(STY9)** genome annotation showed that this genome has 4,701 protein coding sequences (CDS), 38 transfer RNA (tRNA) genes, and 3 ribosomal RNA (rRNA) genes. The annotation included 521 hypothetical proteins and 4,180 proteins with functional assignments Table (3-10). The proteins with functional assignments included 1,282 proteins with Enzyme Commission (EC) numbers, 1,046 with Gene Ontology (GO) assignments, and 898 proteins that were mapped to KEGG pathways. In contrast, **(SalT33)** genome has different numbers, genome annotation showed has 4855 protein coding sequences (CDS), 68 transfer RNA (tRNA) genes, and 4 ribosomal RNA (rRNA) genes. The annotation included 530 hypothetical proteins and 4,325 proteins with functional assignments Table (3-10). The proteins with functional assignments included 1,296 proteins with Enzyme Commission (EC) numbers, 1,059 with Gene Ontology (GO) assignments, and 904 proteins that were mapped to KEGG pathways. However, the annotated characteristics of the studied *S. enterica* strains (STY9 , SalT33) strains were similar with slightly difference in number and function of genes. The annotated features are summarized in Table (3-10).

Table (3-10): Annotated Genome Features of Salmonella enterica subsp. enterica serovar Typhimurium STY9 and Salmonella enterica subsp. enterica serovar Typhi SalT33

Features	**STY9**	**SalT33**
CDS	4,701	4855
Repeat Regions	63	4
tRNA	38	68
rRNA	3	4
Hypothetical proteins	521	530
Proteins with functional assignments	4,180	4,325
Proteins with EC number assignments	1,282	1,296
Proteins with GO assignments	1,046	1,059
Proteins with Pathway assignments	898	904

CDC= coding sequence, EC= Enzyme Commission , GO= Gene Ontology

According to above results, SalT33 strain resulted more CDS than STY9. Moreover, genome of SalT33 strain possessed more genes encoded for tRNA and rRNA than other studied strains. This is small variety in numbers among the studied genomes could be related to the different abilities of gene gain or lose for each genome that effected by the selective pressure in the environmental conditions. Generally, number of CDS that identified in this study were more than CDS of other studies (Ong *et al*., 2012) ,while number of RNAs were lower than another study (Ong *et al*., 2012) . Moreover, the higher number of hypothetical proteins were identified in SalT33 strain rather than STY9 strain indicating to gain new genes with unknown functions. These results may be good answer to interpret the predominance of *Salmonella enterica* subsp. enterica serovar Typhi (SalT33) strain as a causative agent for typhoid fever disease in Babylon Province. The results of this study similar to other study by (Mohakud *et al*., 2022) .With regard to proteins with functional assignments in all studied strains, the results of this study revealed that most of these proteins were identified as enzymes by Enzyme Commission database more than other proteins that were mapped to KEGG pathways or Gene Ontology proteins. In conclusion, alignment of genome sequences of the studied strains with these different resources of genome annotation databases represent the extend of function variety among the studied strains.

3.5.3.2 General proteome comparison among *Salmonella enterica* strains (STY9 , SalT33)

Multiple proteome comparison was performed in this study to identify the presence or absence of the genetic elements and proteins among the studied *Salmonella enterica* genomes (STY9 , SalT33) and the reference genome (NC_003197.2 , NC_003198.1) respectively . All genomes of the studied strains alignment to the reference genome to draw circular heat map depend on uni- and bidirectional best-BLAST hit similarity. The results of this comparison revealed that most of proteins absence occurred at SalT33 compared with STY9. In addition, the higher rates of protein sequence with poor identity or lacking of proteins in contrast to reference proteome were identified in SalT33 strain figure (3-16) (3-17).

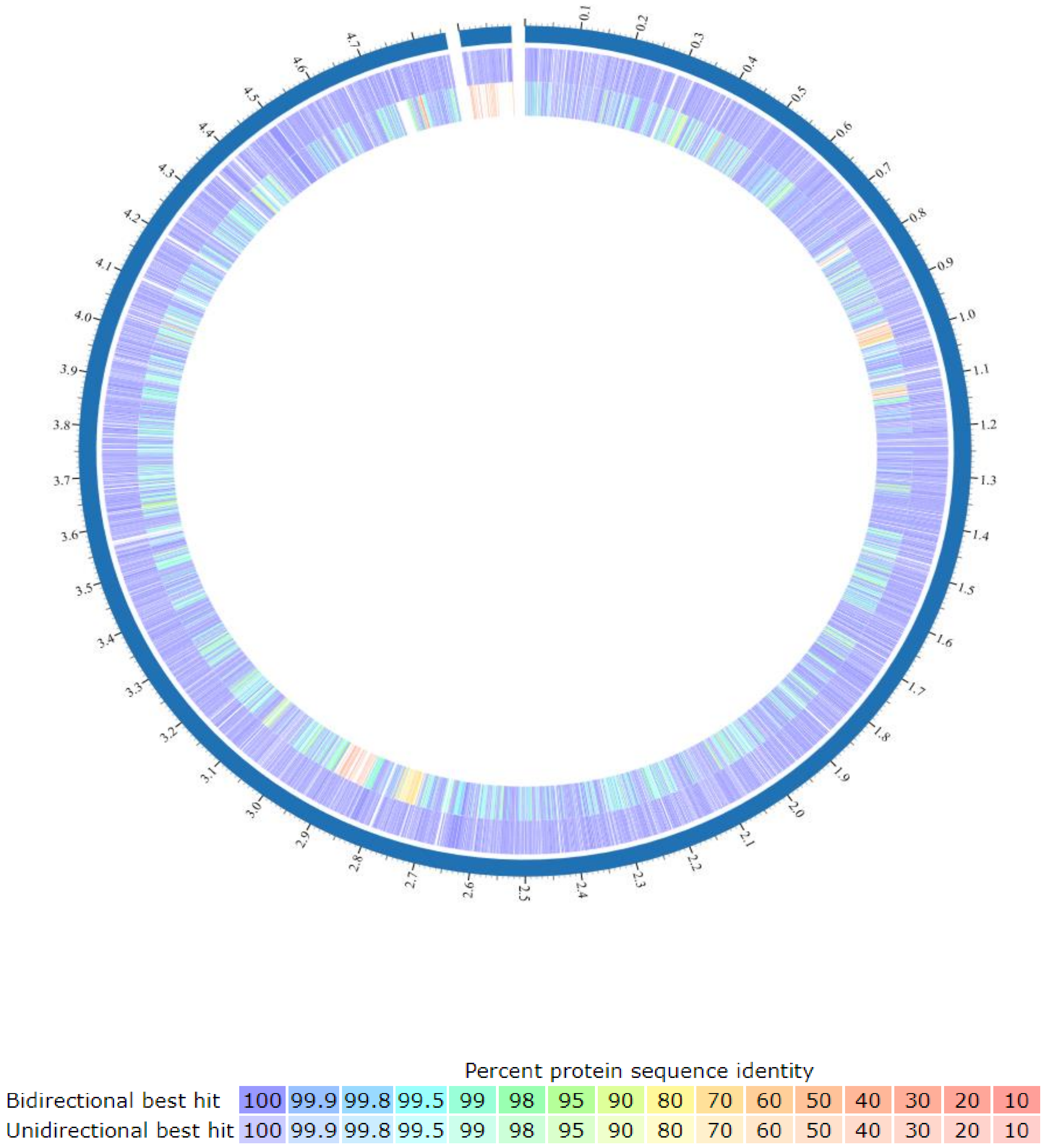


Fig (3-16): Comparative Proteome analysis of studied STY9 isolate against the Reference genome (NC_003197.2).

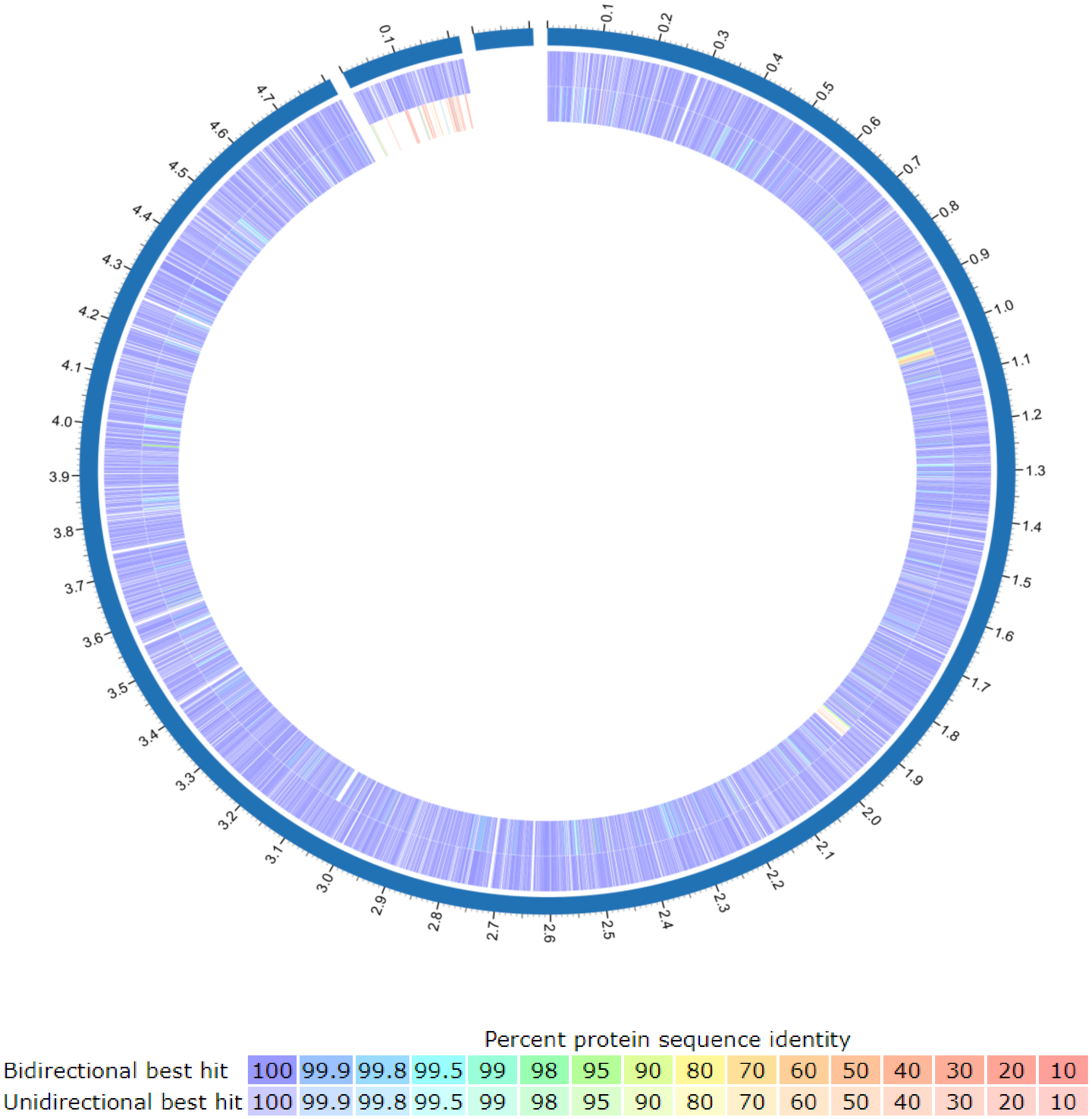


Fig (3-17): Comparative Proteome analysis of studied SalT33 isolate against the Reference genome (NC_003198.1).

3.5.3.3 Identification of subsystem groups in *Salmonella enterica* genomes (STY9 , SalT33)

According to protein functions, this study categorized of the annotated proteins into subsystem groups. These subsystems included Metabolism , Protein Processing, Energy, Cellular Processes, Membrane Transport, Stress Response, Defense, Virulence, DNA Processing, RNA

Processing, Cell Envelope, Regulation and Cell Signaling, and Miscellaneous Groups. Regarding to STY9 and SalT33 genomes, the results of this study showed that 50% of the whole proteome was aligned into subsystems groups while 50% of these proteins not categorized into any subsystems group indicating that they have specific functions.

With regard to STY9 genome, the results of this study revealed that the number of genes assigned to subsystem groups were 993 Metabolism , 371 Energy, 267 Protein Processing , 197 Stress Response, Defense, Virulence, 174 Membrane Transport, 148 Cellular Processes , 125 Cell Envelope, 120 DNA Processing, 76 RNA Processing, 59 Miscellaneous proteins and 32 Regulation and Cell Signaling . Similarly, SalT33 genome possessed 967, 349, 259, 194, 189, 143, 128, 115, 76, 150, 33 Regulation and Cell Signaling respectively. However, all studied *Salmonella enterica* genomes revealed that the higher number of genes were assigned to metabolism subsystem, then to protein processing and energy subsystems suggesting that the larger part of the whole proteome of *S. enterica* are belongs to Bacterial survival and that may explain why this type of bacteria has ability to survive in different habitats. Moreover, all studied genomes had significant number of genes pertaining to pathogenicity and virulence of *S. enterica* , where 197 (STY9), 194 (SalT33) genes were found to be related for bacterial Stress Response, Defense and Virulence. That in turn interpreting the huge ability of *S. enterica* to cause endemic Salmonella enterica disease.

In addition, this study noted that number of genes in the studied subsystem groups was slightly different between STY9 and SalT33. Where, several extra genomic elements identified in STY9 and SalT33 genomes. With more details, an overview of the subsystem groups assigned to the studied genomes and distribution of the subsystem groups with subsystem coverages of each genome were illustrated in figures (3-18), (3-19).

The functional annotation of genomes was investigated. A subsystem is a set of proteins that implements biological processes or structural complexes, and annotations include an analysis of the subsystems unique to the genome. Study by (Mohakud *et al*., 2022) were shown the complex processes include Metabolism (33.75%), followed by Protein processing (13.70%) and Stress response, Defense, and Virulence (13.38%). Further, we elaborated ’Metabolism’ by dividing it into three sections as sequential metabolism and genetic material metabolism. Most of the identified genes were supposed to be involved in carbohydrate metabolism and notably, many genes were predicted to show association with virulence and pathogenesis.

There are no researches comparing among subsystems of *S. typhimurium* and *S. typhi* strains simultaneously. This study may be the first study that identifies the minimum differences among *S. enterica* (*S. typhimurium* and *S. typhi*) types in Iraq. However, understanding of protein functions of *S. enterica* genomes contributes to explain the different mechanisms of organism responses to their natural environments that in turn providing enough solutions to control on Salmonella outbreak. In this study, it was found the most of genes ara involved in metabolism system , they may represent genes important for nutrient acquisition within the host.

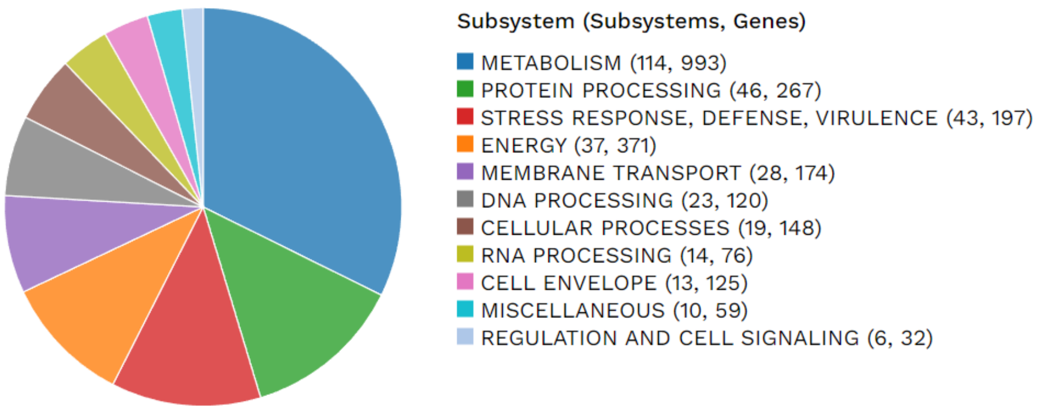


Figure (3-18) Subsystem Analysis of Salmonella enterica subsp. enterica serovar Typhimurium (STY9)

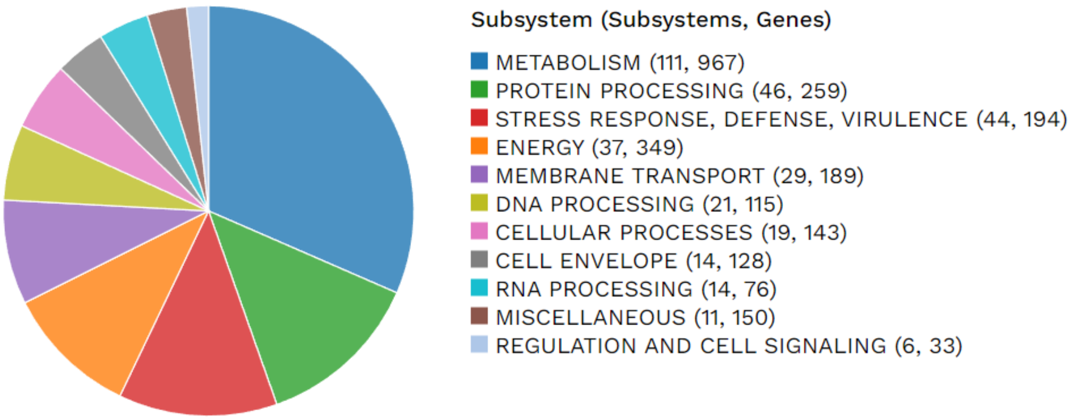


Figure (3-19) : Subsystem Analysis of Salmonella enterica subsp. enterica serovar Typhi (SalT33)

3.5.3.4 Identification of the specialty genes of genome annotations for *S. enterica* serotypes (STY9 and SalT33).

As part of this study, all genes that are specific to infectious *Salmonella* disease were identified in the whole genomes of studied *S. enterica* sp. strains (STY9 and SalT33). These genes have homology to known antibiotic resistance genes, drug targets, transporters and virulence factors. The findings of this investigation showed the presence of 134 antibiotic resistance genes, 405 drug target genes, 702 transporter genes and 793 virulence factor genes at the whole genome of STY9 strain. Regarding SalT33 strain, the specialty genes calculated as the following 119 antibiotic resistance genes, 403 drug target genes, 706 transporter genes and 783 virulence factor genes , as shown in table (3-11) that summarized numbers of the specialty genes in the studied *S. enterica* genomes. For more illustration, position and distribution of the specialty genes across the whole genome of studied *S. enterica* (*S. typhimurium* and *S. typhi*) genomes were visualized by Circos representation in figures (3-20) (3-21).

Many of the genes annotated for both samples in have homology to known transporters, virulence factors, drug targets, and antibiotic resistance genes. The number of genes and the specific source database where homology was found is provided table (3-11).

Table (3-11) Specialty Genes of Salmonella enterica subsp. enterica serovar Typhimurium STY9 and Salmonella enterica subsp. enterica serovar Typhi SalT33

Specialty	**STY9**	**SalT33**
Antibiotic Resistance	134	119
Drug Target	405	403
Transporter	702	706
Virulence Factor	793	783

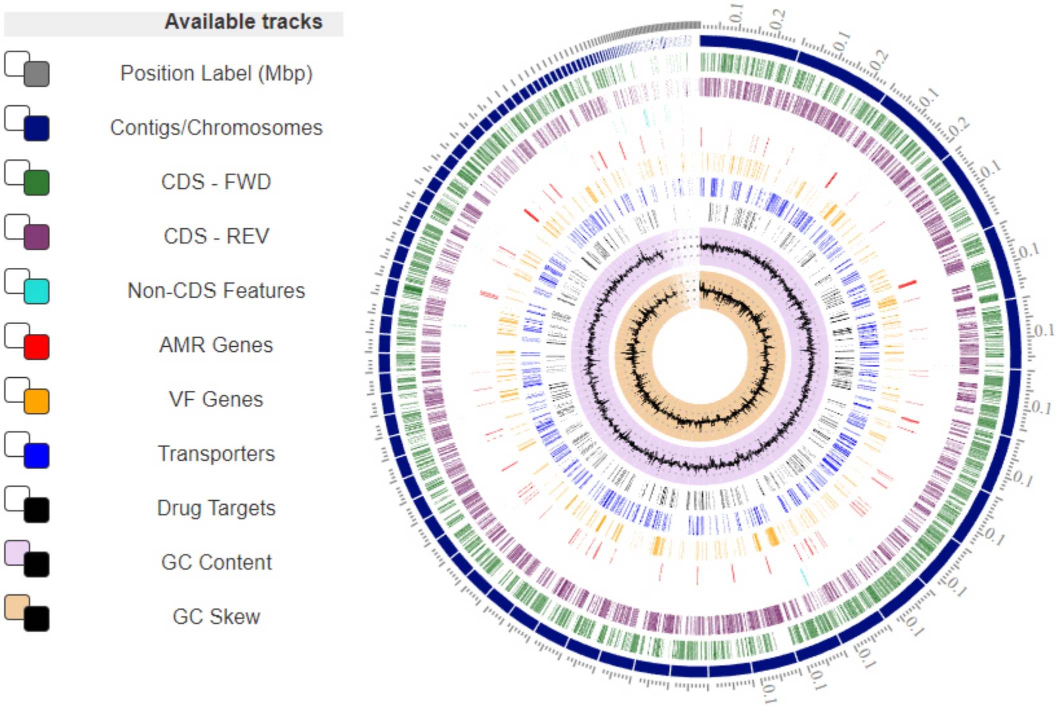


Figure (3-20) Circos representation display of the distribution of the Specialty Gene of genome annotation for STY9. This includes, from outer to inner rings , Position label , the contigs , CDS on the forward strand, CDS on the reverse strand , Non-CDS Features , CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, CDS with homology to know Transporter , CDS with homology to know Drug targets , GC content and GC skew.

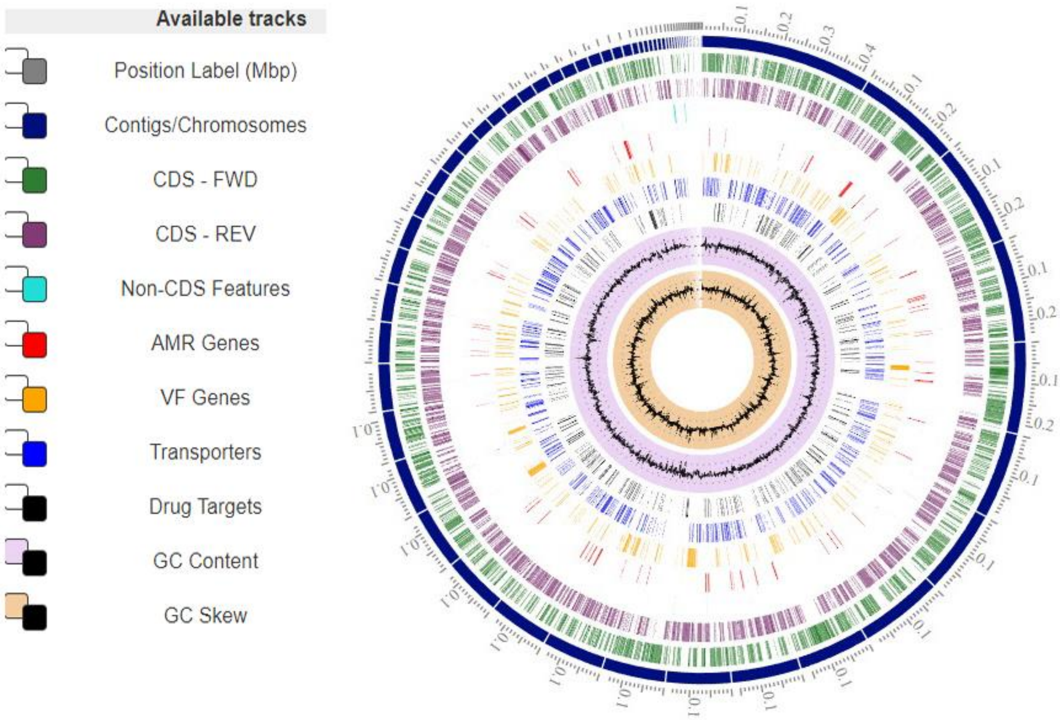


Figure (3-21) Circos representation display of the distribution of the Specialty Gene of genome annotation for SalT33. This includes, from outer to inner rings , Position label , the contigs , CDS on the forward strand, CDS on the reverse strand , Non-CDS Features , CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, CDS with homology to know Transporter , CDS with homology to know Drug targets , GC content and GC skew.

According to the numbers of specialty genes that described above, this study determined a small variety in the numbers of specialty genes among the studied genomes. In exception of Drug Target genes ,Transporter, genome of SalT33 strain showed less number of specialty genes in comparison with STY9 strain. For instant, only 783 Virulence Factor genes were identified in SalT33 strain while STY9 genomes generated 793 genes . This result may explain why STY9 strain are in particular interest with sever disease that has epidemic potential rather than typhi-like disease caused by SalT33 strain that do not has epidemic potential. Additionally, this study noted that STY9 and SalT33 strains showed slightly similar numbers of transporter and Drug Target genes, but these strains showed slightly variety in numbers of antibiotic resistance and Virulence factor genes. For example, the higher number of antibiotic resistance genes was identified in STY9 genome. These findings may refer to the mechanisms of STY9 and SalT33 strains to cause disease were slightly similar but they have different abilities to antibiotic resistance. However, identification of number, distribution and position of specialty genes in this study may facilitate understanding biology of *S. enterica* sp. and provide important features toward studying their pathogenesis at genomic level.

3.5.3.4.1 Annotation of the Antimicrobial Resistance Genes (AMR) across the genomes of *Salmonella enterica* strains (STY9 and SalT33)

This type of specialty genes was annotated to identify type of antimicrobial resistance genes and corresponding antimicrobial resistance mechanisms. The results of this study revealed the presence different mechanisms for antibiotic resistance at all studied genomes. The antimicrobial resistance mechanisms that identified in this study included antibiotic activation enzyme, antibiotic inactivation enzyme, antibiotic target in susceptible species, antibiotic resistance gene cluster ,cassette , or operon , efflux pump conferring antibiotic resistance, gene conferring resistance via absence, protein altering cell wall charge conferring antibiotic resistance, regulator modulating expression of antibiotic resistance genes and antibiotic target protection protein. A summary of the AMR genes annotated in the studied genomes and corresponding AMR mechanisms is provided in tables (3-12) (3-13).

Table(3-12) Antimicrobial Resistance Genes for STY9

AMR Mechanism	Genes
Antibiotic activation enzyme	KatG
Antibiotic inactivation enzyme	AAC(6')-Ic,f,g,h,j,k,l,r-z
Antibiotic resistance gene cluster,cassette,or operon	MarA, MarB, MarR
Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target protection protein	BcrC
Efflux pump conferring antibiotic resistance	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, MexPQ-OpmE, OprM/OprM family, SugE, Tet(B), TolC/OpmH
Gene conferring resistance via absence	gidB
Protein altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Regulator modulating expression of antibiotic resistance genes	AcrAB-TolC, EmrAB-TolC, H-NS, OxyR

Table(3-13) Antimicrobial Resistance Genes for SalT33

AMR Mechanism	Genes
Antibiotic activation enzyme	KatG
Antibiotic inactivation enzyme	AAC(6')-Ic,f,g,h,j,k,l,r-z
Antibiotic resistance gene cluster,cassette,or operon	MarA, MarB, MarR
Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target protection protein	BcrC
Efflux pump conferring antibiotic resistance	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, OprM/OprM family, SugE, TolC/OpmH
Gene conferring resistance via absence	gidB
Protein altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Regulator modulating expression of antibiotic resistance genes	AcrAB-TolC, EmrAB-TolC, H-NS, OxyR

However, the absence or presence AMR genes could be related to gene loss or gain mechanisms in *S. enterica* genome. According to this mechanism, STY9 genome possessed more genes involved in this mechanism than SalT33, where MexPQ-OpmE and Tet (B) family genes identified in STY9 genome.

Theoretically, this study found that all other AMR genes may provide antibiotic resistance towards one or multiple drugs of the following antimicrobial classes: Fluoroquinolones, Elfamycins, Aminoglycosides, Cycloserine, Isoniazid, Peptide antibiotics, Triclosan, Bicyclomycins, Diaminopyrimidines, Fosfomycin, Fosmidomycin, Fusidic acid, Mupirocin, Penams, Cephamycins, Cephalosporins, Tetracyclines, Carbapemes, Beta-lactam antibiotics, Rifamycins, Mupirocin compounds , Sulfonamides and other antibiotics .

The results of this study correspond to results by (Mohakud *et al*., 2022) .The Genome Annotation Service provided AMR genes detection method, which utilizes collection of representative AMR gene sequence variants and assigns .To each AMR gene, this service assigns functional annotation, comprehensive antibiotic resistance mechanism, different classes of drugs and occasionally, a specific antibiotic to which it confers resistance. It notes that the presence of AMR-related genes (even full length) in a given genome directly implies an antibiotic-resistant phenotype. It is essential to consider specific AMR mechanisms, especially regulator modulating expression, efflux pump conferring, and target in susceptible categories.

The genetic characteristics of several mutations in highly resistant strains could contribute to the survival and dissemination of bacteria and would benefit their environmental stress response.

In this study, we have shown that WGS is an excellent tool for accurately predicting antimicrobial resistant phenotype in human, multiple *Salmonella* serovars, as WGS predictions and phenotypic resistance matched well with high sensitivity and specificity in our study. AMR prediction could be beneficial and can definitely enhance the monitoring of AMR strains and determinants detected in humans .

Paradoxically, the presence of AMR-related genes in this study on the studied *S. enterica* genomes does not directly infer antibiotic resistant phenotype, where it is important to consider the absence/presence of SNP mutations at these genes conveying resistance (Davis *et al*., 2016).

Of interest, gene mechanisms of tetracycline resistance including the efflux genes, the ribosomal protection and enzymatic genes were suggesting a possible ecological role for specific wide spread of tetracycline resistance (Cadena *et al*., 2018).

The increasing cases reported globally, especially the emergence of multidrug-resistant (MDR) Salmonella have a huge impact on the global health population (Yap *et al*., 2014). WGS has become a popular method of choice for Salmonella studies due to its high sensitivity and specificity for tracking and characterizing the transmission of *S. Typhi* (Eng *et al*., 2015), and for the prediction of antimicrobial resistance phenotypes, such as ampicillin, chloramphenicol, co-trimoxazole, tetracycline and ceftriaxone(Klemm *et al*., 2018).

3.5.3.4.2 Annotation of the Virulence Factor genes across the genomes of *Salmonella enterica* strains (STY9 and SalT33)

Virulence factors might contribute to the pathogenicity and host restriction of Salmonella, but the underlying mechanism is not yet fully understood. Although the genome sequences of several host specific and broad-host Salmonella strains are available, the genetic basis involved in the host restriction of *Salmonella* remain unclear.

In this study, the main Virulence Factors involved in the pathogenesis of *S. enterica* were identified across the whole genome of studied *S. enterica* sp. strains (STY9 and SalT33). This type of specialty genes included Adherence, Toxins, Invasion and other virulence factors.

In addition, chromosomal location of each virulence factor was determined. The findings of this study revealed the presence five types of adherence factors in STY9 , while only four adherence factors were identified on SalT33 genome. The shared adherence factors among all studied genomes were adherence (1) , Adherence, Fimbrial (23), Adherence, Nonfimbrial (2) , Adherence, Nonfimbrial, Autotransporter (3) , while Adhesion , Invasion , Virulence ,Defense against host immune response (1) genomes , as shown in Table (3-14).

Table (3-14) Distribution of the Virulence Genes of Salmonella enterica subsp. enterica serovar Typhimurium STY9 and Salmonella enterica subsp. enterica serovar Typhi SalT33

Virulence	STY9 Virulence type (gene number)	SalT33 Virulence type (gene number)
Adherence	Adherence (1) Adherence, Fimbrial (23) Adherence, Nonfimbrial (2) Adherence, Nonfimbrial, Autotransporter (3) Adhesion,Invasion,Virulence,Defense against host immune response (1)	Adherence (1) Adherence, Fimbrial (12) Adherence, Nonfimbrial (5) Adherence,Nonfimbrial,Autotransporter (3)
Biofilm & Capsule	Biofilm (8)	Biofilm (7) Capsule (9)
Invasion	Invasion (13) Invasion, Chaperone (1) Invasion, Serum resistance (1) Invasion, Type III secretion (3) Invasion, Virulence (4) Invasion, Virulence, Secretion, Avoidance of host defense (1)	Invasion (12) Invasion,Chaperone (1) Invasion,Serum resistance (1) Invasion,Type III secretion (3) Invasion,Virulence (4) Invasion,Virulence,Secretion,Avoidance of host defense (1)
Cell-to-cell spread & Immune evasion	Cell motility (12) Cell-to-cell spread (3)	Cell motility (12) Cell-to-cell spread (4) Immune evasion (9)
Intracellular survival	Intracellular survival and replication (14) Intracellular survival and Secretion system effectors (3) Inhibition of phagolysosome formation (2)	Intracellular survival and replication (15) Intracellular survival and Secretion system effectors (2) Inhibition of phagolysosome formation (2)

Toxin	Endotoxin (3) pore-forming toxin (1)	Endotoxin (3) pore-forming toxin (1) Cytolethal distending toxin DNaseI (1)
Secretion systems	Type I, Type II and other Secretion system (21) Type III secretion system (81)	Type II and other Secretion system (16) Type III secretion system (84)

Here , we hypothesized that the level of virulence , as measured by the Salmonella burden in cells and tissues using different models of infection may be predicted as high or low when combining WGS and phylogeny studies for the presence or absence of gene association with virulence .

The absence of Adhesion , Invasion , Virulence ,Defense against host immune response in SalT33 strain of this study could be related to several evolutionary events such as genomic deletion or insertion.

Regarding Invasion factors and Toxins , this study identified the main factors that responsible to gastroenteritis or typhoid -like infection. According to the whole genome annotation of S. typhimurium STY9, this study revealed the presence of the following Invasion factors and Toxins : Invasion (13) ,Invasion, Chaperone (1) , Invasion, Serum resistance (1) , Invasion, Type III secretion (3) , Invasion, Virulence (4) Invasion, Virulence, Secretion, Avoidance of host defense (1) , Endotoxin (3) and pore-forming toxin (1). In contrary, the whole genome annotation of SalT33 strain showed Invasion factors and Toxins : Invasion (12) ,Invasion, Chaperone (1) , Invasion, Serum resistance (1) , Invasion, Type III secretion (3) , Invasion, Virulence (4) Invasion, Virulence, Secretion, Avoidance of host defense (1) , Endotoxin (3) , pore-forming toxin (1) in addition , Cytolethal distending toxin DNaseI (1) identified in SalT33 genome , while this gene was absent in STY9 .

The existence of Type I, Type II and other Secretion system alongside with Type III secretion system may demonstrate the high ability of STY9 strain to survive and cause diarrheal disease in the same time.

Multiple virulence genes were identified among the several Salmonella serovars across different sources by WGS . These genes have been described to be involved in several processes important for Salmonella transmission and infection, including adhesion, type III secretion system (T3SS), host recognition/invasion, filamentous formation, magnesium uptake, iron acquisition, and regulation of stress factors.

Among virulence factors, fimbriae have a major role in pathogenesis and a source of diversity for Salmonella serovars. This factor is one of the most common adhesion systems and are differentially expressed and found in a specific pattern among each serovars (Dufresne & Daigle, 2017).

The cdtB encodes the typhoid toxins of *S. Typhi* and is not reported from a wider distribution among non-typhoidal Salmonella serovars (NTS (Mezal *et al*., 2013). This data suggested that the cdtB toxin may contribute to the pathogenicity in human .

A comparison of the *S. enterica* serovar *Typhimurium* (*S. Typhimurium*) strain LT2 and the *S. Typhi* strain CT18 revealed that both species shared approximately 89 percent of genes, with ≈ 480 genes found to be unique to *S. Typhimurium*, and ≈ 600 genes found to be unique to *S. Typhi* (McClelland *et al*., 2001). According to Sabbagh *et al*., (2010), each species of *Salmonella* evolves through horizontal gene transfer or gene degradation mechanisms. In addition, genome sequencing analysis has resulted in discovering over 200 pseudogenes, many relating to genes believed to contribute to *S. Typhi* virulence. This genetic degradation can lead to *S. Typhi's* human-restricted host range which can be found in phages, SPI genes, fimbriae and other virulence factors.

A global comparison study of *S. enterica* isolates demonstrates the emergence of closely related isolates during a long period and underline the genetic homogeneity of this serovar. The virulence of *Salmonella enterica* depends on diverse assortment of genes which are required for adhesion, invasion, intra-cellular survival, and replication. These genes are located on various elements of the genome including *Salmonella* pathogenicity islands (SPIs)(Zhao *et al*., 2020).

Gene gain or loss is generally associated with Salmonella virulence and host adaptation. For example, the expression of *GtgE*, a unique type III secretion system effector protein from the broad-host *S. Typhimurium*, allows *S. Typhi* to survive and replicate within mouse macrophages and tissues(Spanò & Galán, 2012). The earliest step in *Salmonella* pathogenesis is its attachment to the host cell, which is typically mediated by fimbriae that target particular cell types. The *Tcf* is a chaperone-usher fimbria, which may play a role in the host specificity of typhoidal serotypes. Although the tcf operon is present in at least 25 NTS serotypes, it contributes differently to the virulence of distinct NTS serotypes and exhibits a varying expression profile in different Salmonella serovars(Azriel *et al*., 2017).

Recently, WGS has been successfully used in our previous studies to assess the genetic diversity of *S. Enteritidis* and *Salmonella Typhimurium* serovars(Ktari *et al*., 2020). The use of these technologies has greatly enlarged our view of the genetic diversity of this bacterium. Furthermore, it produces an opportunity to provide more genetic information to study all genetic determinants such as virulence markers, antimicrobial resistance genes, mobile elements, bacteriophages and to determine genomic changes associated with pathogenicity and antibiotic resistance.

3.5.4 Variants calling

As a part of the whole genome analysis of *S. enterica* strains (STY9 and SalT33), genetic differences (variants) were called between the studied genomes and reference genome for identifying the existence of a single-nucleotide polymorphism (SNPs), Insertions, Deletions and Base changes. In addition, this study identified types of SNPs either Transition or Transversion variants at the whole genome of studied *S. enterica* strains. Moreover, this study determined number, impact and percentages of each variant annotation type.

3.5.4.1 Variants calling for *Salmonella enterica* stains (STY9 and SalT33)

According to the general variants count, the results of this study showed the existence of 25608 variants on STY9 genome in comparison with the reference genome. With regard to SalT33 genome, 680 variants were identified compared with reference genome. Interestingly, STY9 genome generated the higher number of variants in contrast to SalT33 genome as shown in table (3-15).

Furthermore, the results of this study revealed that SNPs variants covered the higher number of variant counts in all studied genomes (STY9 and SalT33), where 25355 (99.01%) of STY9 variants identified as SNPs while only 134 (0.53%) and 119 (0.46%) of the total number of STY9 variants detected as Insertions and Deletions. Similarly, of a total of 680 variants determined in SalT33 genome, SNPs comprised 639 (93.97%) while only 20 (2.95%) Insertions and 21 (3.08%) Deletions were identified. Number and percentages of variants summarized in table (3-15)

Table (3-15): Comparison of Salmonella enterica subsp. enterica serovar Typhimurium STY9 genome with reference genome to identify variants (Nucleotide Polymorphism, Indels)

Sample	**Data**	**Nucleotide Polymorphism**	**Indels**		**Total**
		SNP	**Insertion**	**Deletion**	
STY9	**N**	**25355**	**134**	**119**	**25608**
	%	**99.01**	**0.53**	**0.46**	**100**
SalT33	**N**	**639**	**20**	**21**	**680**
	%	**93.97**	**2.95**	**3.08**	**100**

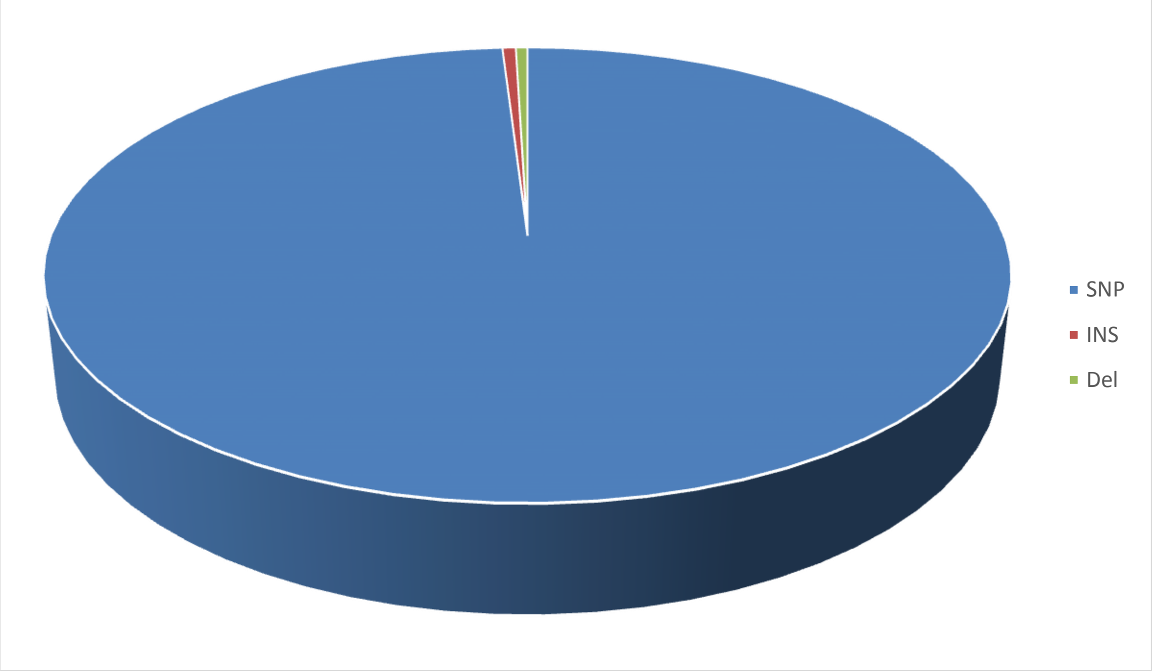


Fig. (3-22): Variants Distribution among Salmonella enterica subsp. enterica serovar Typhimurium STY9 genome in comparison with Reference genome.

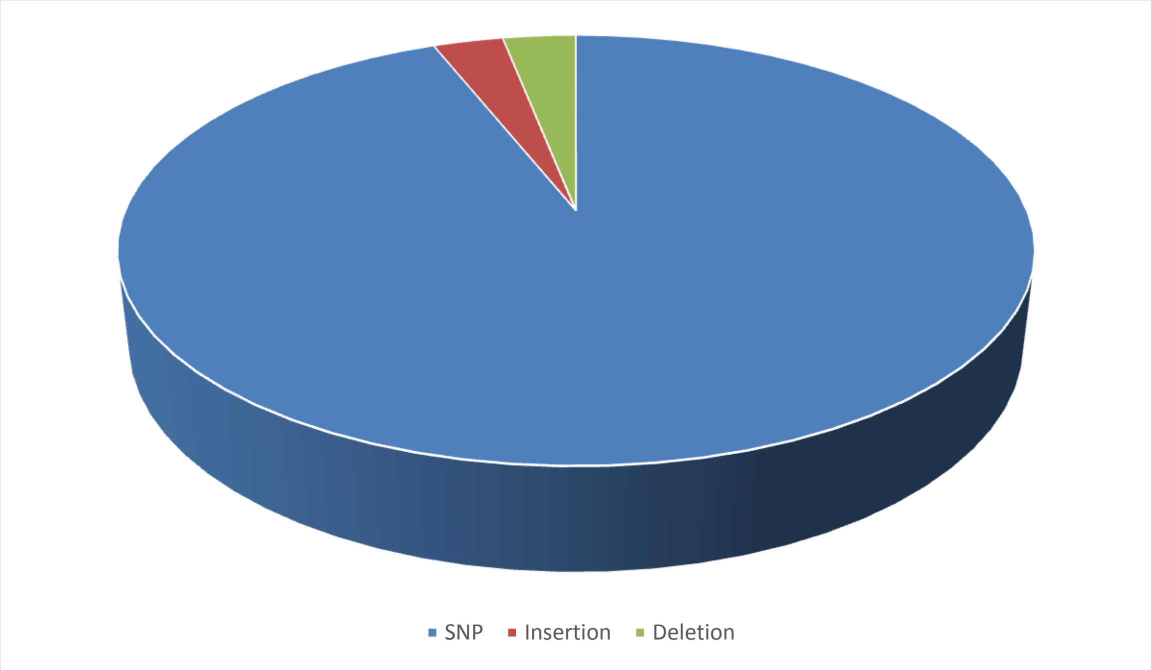


Fig. (3-23): Variants Distribution among Salmonella enterica subsp. enterica serovar Typhi SalT33 genome in comparison with Reference genome.

According to described data above, the general variants count of studied genomes showed STY9 strain was more relative to the reference genome than SalT33 strain , where only 680 variants were identified.This result explains the huge evolutionary events that occurred on STY9 genome that force this strain to be so far from the reference genome lineage, suggesting that STY9 genome evolved independently from this lineage in spite of they have shared ancestry.

Additionally, the findings of this study revealed that the higher rate of genetic variation for the studied genome was related to SNPs more than Insertions and Deletions.

Furthermore, this result documented the presence of very high rate of SNPs in STY9 genome compared with the reference genome. Additionally, this study noted low rates of insertion-deletion (Indels) in all studied genomes. Generally, the high level of SNPs reflects high point mutation rates (Alwi, 2005). Thus, this study presumes that the most common type of genetic variation in the studied *S. enterica* genomes is single-base mutations rather than Indel Mutations.

Genetic variability between serovars occur for a varity of reasons such as point mutations , single nucleotide insertion or deletion .

The effect of genomic changes on translation was variable. The genomic features of SNPs identified, including the SNP position and type, allele length, annotation, coding region and amino acid changes. The number of SNP difference between isolates observed depends on mutation rate and the evolutionary time that has passed. Therefore, the small number of SNP differences observed between our isolates suggests that it is highly likely that these isolates shared a very recent common ancestor.

3.5.4.2 Identification of patterns of nucleotide substitution for studied *Salmonella enterica* genomes (STY9 and SalT33)

After identification of SNPs variants, this study calculated the base change count on every SNPs to identify the type of sequence variation. The results of this study revealed that base substitution among studied *S. enterica* genomes (STY9 and SalT33) was relative, where majority of base substitution on all the studied genomes revealed the following base changes:

Adenine (A) nucleotide was substituted with Guanine (G) or vice versa, and Thymine (T) was substituted with cytosine (C) or vice versa. In more details, the higher percentages of A substitution in STY9 and SalT33 genomes were A → G substitution with 77.74% and 73.91%, respectively, while the higher base-substitution counts for T nucleotide showed T → C substitution with 77.17% and 71.95%, respectively. Moreover, the higher percentages of G substitution noted as G → A substitution with 75.93% and 78.01% respectively. similarly, the most common type of C substitution detected as C → T substitution with 75.36% and 78.03% respectively. General Base change results were summarized in Table (3-16) and illustrated in figures (3- 24 , 3-25) for each studied genome.

Additionally, Transition and Transversion variants were identified to detect patterns of nucleotide substitution. The results of this study revealed that all studied genomes showed high rate of Transition substitution compared with Transversion substitution. With more details, in STY9 and SalT33 genomes, Transition substitution comprised 76.56% and 75.43% , respectively of SNPs variants, while Transversion substitution included only 23.44% and 24.57% , respectively of SNPs variants, as shown in Table (3-17) and figure (3-25).

Table(3-16) General base substitution of STY9 and SalT33 genomes

Genome	***Ref***	**A**			**T**			**G**			**C**		
	Alt	**T**	**G**	**C**	**A**	**G**	**C**	**A**	**T**	**C**	**A**	**T**	**G**
STY9		547	4991	882	564	851	4784	4879	861	686	855	4767	704
		8.52%	77.74%	13.74%	9.10%	13.73%	77.17%	75.93%	13.40%	10.68%	13.52%	75.36%	11.13%
SalT33		17	119	25	17	29	118	110	21	10	28	135	10
		10.56%	73.91%	15.53%	10.37%	17.68%	71.95%	78.01%	14.89%	7.09%	16.18%	78.03%	5.78%

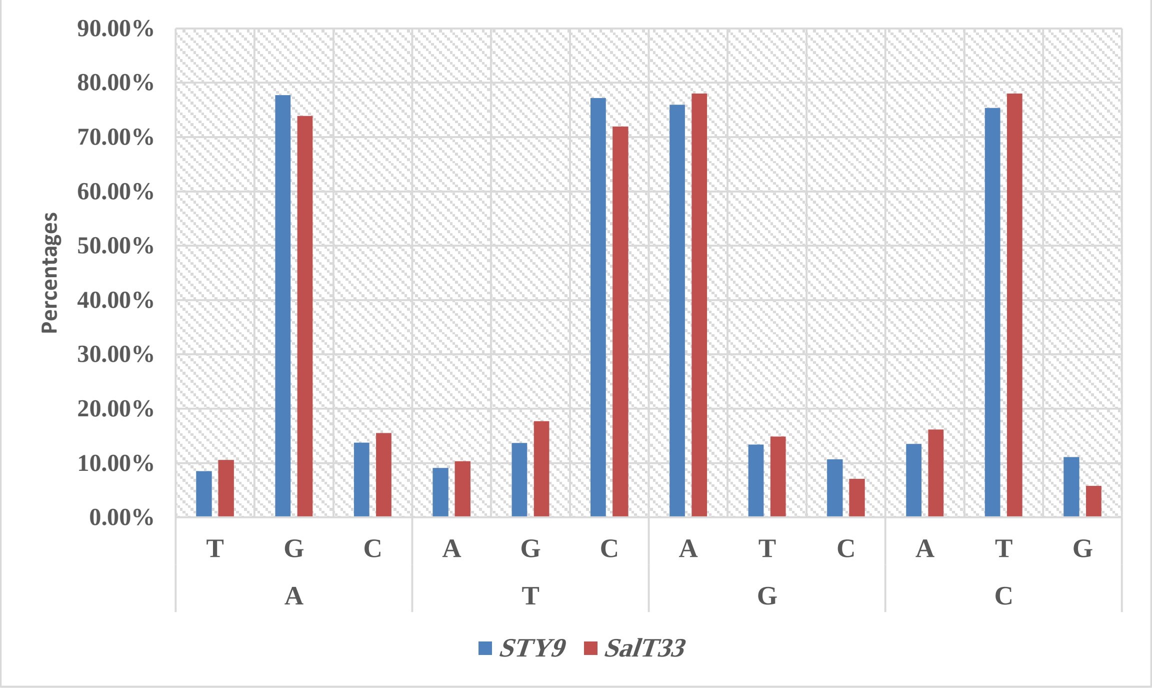


Figure (3-24) Base change results for S. enterica (STY9 and SalT33)

Table: (3-17)Distribution of transitions and transversions among the studied genomes (STY9 and SalT33)

Genome	Total SNP Count	number of transitions	number of transversions
STY9	25355	19413 (76.56)	5942 (23.44)
SalT33	639	482 (75.43)	157 (24.57)

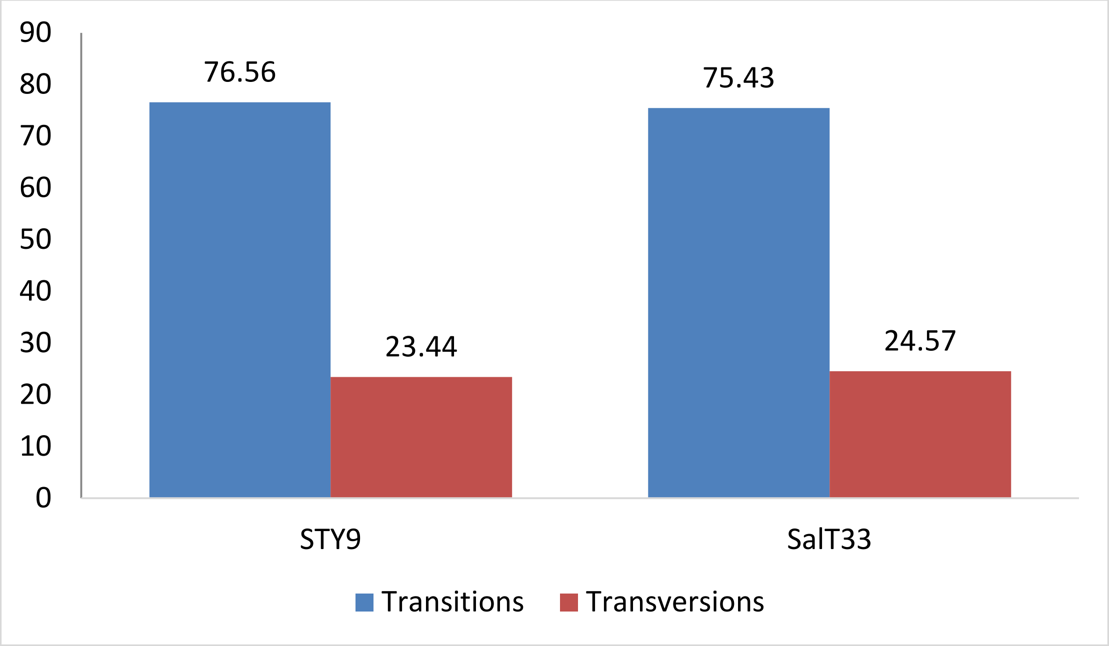


Fig (3-25): Percentages of base substitution type (transitions and transversions) among the studied genomes (STY9 and SalT33)

According above results, there are no significant differences among the studied genomes regarding type of sequence variation or patterns of nucleotide substitution, where all studied genomes showed similar variation patterns. Moreover, the most common patterns of base substitution in all studied genomes were C ↔ T and G ↔ A substitutions. Based on these results, these patterns represent Transition substitution rather than Transversion substitution, where Transition variants comprised the higher percentages in comparison with Transversion variants in all studied genomes after SNPs analysis.

The high rate of transition substitution could be related to absence repair mechanisms that must revert C ↔ T and G ↔ A transitions.

The total number of SNPs observed among the isolates varies depending on serovars, in particular for the most homogenetic ones, the sample size, the time span, and the filtering approaches for SNP detection. The type of the outbreak is also one important variable in terms of within-outbreak SNP differences (Besser *et al*., 2019).

The *S. enterica* genome is thought to have a mutational bias towards G´C base pairs, transitions creating A´T pairs were approximately 10 times more frequent than the reverse mutation, resulting in an overall mutation pressure to lower G+C contents. Transitions occurred at roughly twice the rate of transversions, similar to results from sequence comparisons; however, several individual transversions are more frequent than the least common. Differences among point mutation rates can also affect the overall G+C content of a genome. For example, when C→T transitions occur at a higher rate than T→C transitions, A´T pairs will accumulate at neutral sites, such as non-coding DNA, and degenerate codon positions. By measuring mutation rates for all possible point mutations, we found a highly bias towards the formation of A.T pairs among transitions, but no bias among transversions . Changes in the patterns of mutations within a species can be exposed by reconstructing the substitutions in a phylogenetic context (Hudson *et al*., 2003).

3.5.5 Phylogeny

In the present study, phylogenetic analysis of the studied *S. enterica* sp. genomes (STY9 and SalT33) was performed to determine the closest representative genomes of *S. enterica* and other species. The results of phylogenetic analysis revealed that the closest representative genomes of S. *typhi* genome was *S. enterica* sp. serovar typhi str.CT18 220341.7 , while *S. typhimurium* genome was S. enterica sp. serovar Typhimurium str. LT2 99287.12 . This strain shared high homology with studied STY9 and SalT33 strains , and grouped with the same clade for each one suggesting same clone origin.

Furthermore, the nearest phylogenetic neighbor of *S. enterica* to the studied genomes was *S. enterica* sp. serovars Paratyphi A str ATCC 9150295319.15 and *S. enterica* sp. serovar Typhimurium str. 14028s 588858.6 strain while the furthest neighbor of *Salmonella* was *Salmonella bongori* NCTC 12419 218493.5 strain. Phylogenetic placement of studied genomes illustrated in figure (3-26).

The phylogenetic tree showed that the isolates from this study were tightly clustered together as one group . The *S. enterica* isolates from different infection close genetic relationships , and epidemiology or trace back evidence was provided to establish the connection between these *S. enterica* isolates .

However, identification of the phylogenetic placement of the studied genomes in Babylon Province of Iraq and determination of the closest representative genomes could facilitate understanding evolution of *S. enterica* , that in turn may assist in restoration of control polices and strategies to eliminate *Salmonella* disease in Iraq.

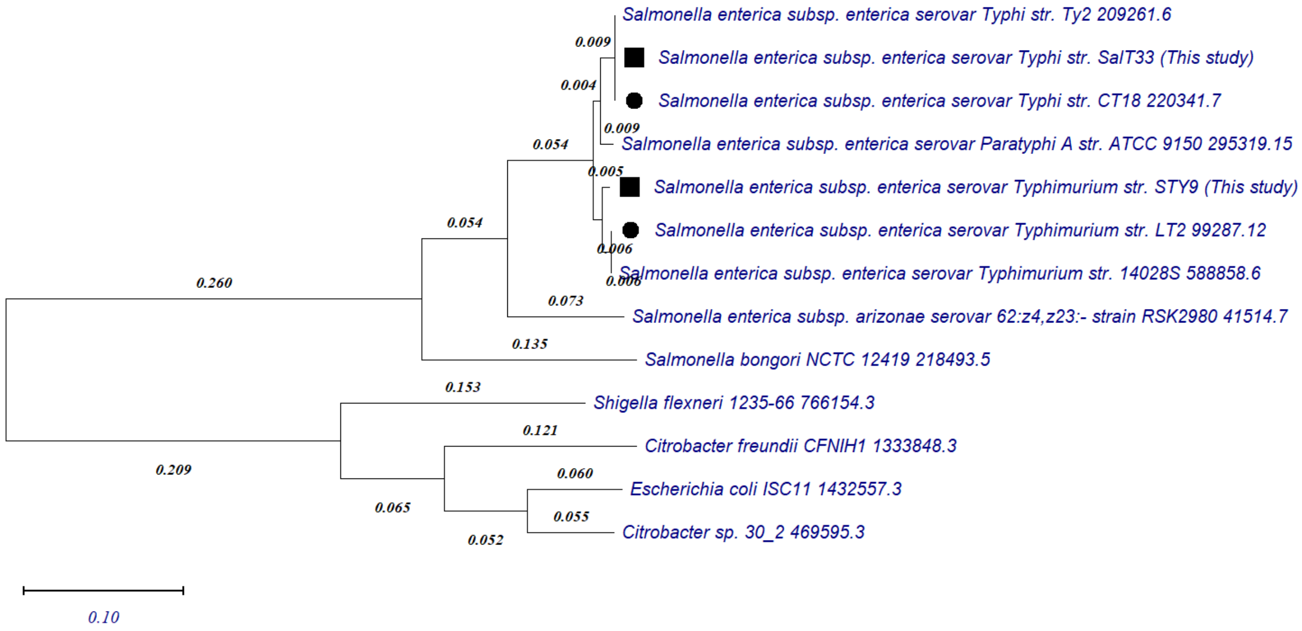


Figure (3-26) Phylogenetic analysis of S. enterica sp. (S. typhimurium and S. typhi) , the closest reference and representative genomes to Salmonella enterica subsp. enterica serovar Typhimurium STY9 and Salmonella enterica subsp. enterica serovar Typhi SalT33 genomes to determine the phylogenetic placement of this genome was determined by Mega X.

The phylogenetic analysis based on WGS-derived SNPs has been shown to provide greater cluster resolution than the gold standard subtyping method, pulsed-field gel electrophoresis (PFGE), resulting in discrimination of outbreak-related human clinical isolates and food or environmental origins (Scaltriti *et al*., 2015).

The phylogenetic clustering is an effective tool that relies on an alignment-free approach for genomic evolution study, the phylogenetic trees acquired are affected by recombinant genes and/or horizontal gene transfer including plasmid , prophage , and other accessory gene contents (van Vliet & Kusters, 2015).

In general, 16 s rRNA sequencing-based phylogenetic- tree analysis is the most frequently used method to classify organisms. Study by (Mohakud *et al*., 2022) were found that the phylogenetic tree analysis, we have identified that *S. enterica* Typhimurium ms202 has 99.9% similarity to *S. enterica* subsp. *salamae* strain DSM 9220 and *Salmonella enterica* subsp. *enterica* strain LT2. The study on comparative analysis indicated a close relationship among these bacteria (Porwollik *et al*., 2002) and suggested the association with disease in humans (Chan *et al*., 2003).From phylogenetic tree analysis, we find that *S. enterica* Typhimurium ms202 is far from *S. bongori* The distinct taxonomic grouping defines the genealogical species concept for *S. enterica* Typhimurium ms202 and emphasizes the understanding of genetic diversity and phylogenicity among *S. enterica* serovars (Timme *et al*., 2013).

Conclusions

&

Recommendations

Conclusions:

The study has reached at the following conclusions:

1. Detection of *Salmonella enteritidis* , *Salmonella typhimurium* and *Salmonella typhi* using specific primer SE1472298-2 , STM4497 and gene O antigen synthesis *tyv* respectively , more specific than biochemical test.
2. *Salmonella enterica* isolates were sensitive to almost types of antibiotics used in this study.
3. The indication of genetic diversity among clinical *Salmonella enterica* isolates which is constitutes with high level of polymorphism can be certified with study of genotyping method (CRISPR) that gave a significant assumption for evolutionary ancestral gene .
4. The evolution of *Salmonella enterica* that has diverse host specificity , linking various regulatory networks with the CRISPR cas system.
5. CRISPR typing is a valuable molecular subtyping method for tracking different source of S. enterica and its useful tool to reveal the phylogenetic relation ships among these isolates .
6. SNP comprised the majority of varients in comparison with insertion and deletion , most of these SNPs are transition substitution (C ↔ T) and (G ↔ A) substitution pattern.
7. STY9 genome generated the higher number of variants in contrast to SalT33.
8. WGS is a helpful tool to assess the phylogenetic relations among multiple serotypes, AMR and virulence gene evaluation and assist in the molecular epidemiological studies.
9. The application of next-generation sequencing (NGS) to the whole-genome sequencing of infectious illnesses, particularly S.

Typhi and S. typhimurium genomes, has permitted a greater understanding of the pathogen’s evolutionary trends and its pathogenesis.

Recommendations :

Depending on the finding of this study the recommended objective include:

1. Direct and rapid identification of *S. enterica* in clinical samples through using molecular technique because of the high degree of specificity and sensitivity obtained in this study .
2. Using Real-time PCR to detect pathogen as the main causative agent depending on the copy number.
3. Comparative study the pathogenesis between *Salmonella* strains which isolated from human and animal .
4. Further studies are needed to study the whole transcriptome of *S. enterica*.
5. Studying the whole genome of *S. enterica* isolated from all Iraqi provinces.
6. Further studies can determine the exact role of CRISPR-cas with virulence factor and antibiotic resistance of *S. enterica*

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Appendix

Appendix 1 : Identification Information of *Salmonella enterica* Card Type: GN

bioMérieux Customer: System #:	Laboratory Report	Printed May 21, 2022 11:36 CDT Printed by: Labadmin

Patient Name:
Isolate: 201-1 (Qualified)

Patient ID:
Bench: swab

Card Type: GN Bar Code: 2410662403333784
Testing Instrument: 000013B066CF (The Imam Al-Sadiq Teaching -Hospital)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0017610741566210
Organism Quantity:
Selected Organism: Salmonella enterica ssp enterica

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2410662403	**Expires:**	Jul 25, 2024 13:00 CDT
	Completed:	May 20, 2022 17:14 CDT	**Status:**	Final	**Analysis Time:**	4.82 hours
Organism Origin	VITEK 2					
Selected Organism	95% Probability		**Salmonella enterica ssp enterica**			
	Bionumber:	0017610741566210			**Confidence:**	Very good identification
SRF Organism						

Analysis Organisms and Tests to Separate:

Salmonella group
Salmonella spp
Salmonella ser.Paratyphi B
Salmonella ser.Typhimurium
Salmonella ser.Paratyphi C
Salmonella ser.Enteritidis
Salmonella enterica ssp enterica

Analysis Messages:

Confirm by serological tests

Contraindicating Typical Biopattern(s)

Salmonella group	URE(1),

bioMérieux Customer:

Laboratory Report

Printed May 21, 2022 11:36 CDT
Printed by: Labadmin

System #:

Patient Name:
Isolate: 201-1 (Qualified)

Patient ID:
Bench: swab

Card Type: GN Bar Code: 2410662403333784

Testing Instrument: 000013B066CF (The Imam Al-Sadiq Teaching -Hospital)

Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0017610741566210
Organism Quantity:

Selected Organism: Salmonella enterica ssp enterica

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	-	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Appendix 2 : Antibiotic susceptibility test Card Type: AST-GN76

Printed May 21, 2022 11:37 CDT

bioMérieux Customer:

Microbiology Chart Report

The Imam Al-Sadiq Teaching -Hospital

Patient Name:

Patient ID:

Location:

Physician:

Lab ID: 202

Isolate Number: 1

Organism Quantity:

Selected Organism : Salmonella enterica ssp enterica

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	4.10 hours	**Status:** Final
Selected Organism	95% Probability	**Salmonella enterica ssp enterica**	
	Bionumber:	0015610661546210	
ID Analysis Messages			

Susceptibility Information	**Analysis Time:** 10.18 hours			**Status:**	Final
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
ESBL			Imipenem	<= 0.25	S
Ampicillin	>= 32	R	Amikacin	<= 2	*R
Piperacillin/Tazobactam	<= 4	S	Gentamicin	<= 1	*R
Cefazolin	<= 4	*R	Ciprofloxacin	<= 0.25*	S
Cefoxitin	<= 4	*R	Levofloxacin	0.5	S
Ceftazidime	<= 1	S	Tigecycline	<= 0.5	S
Ceftriaxone	<= 1	S	Nitrofurantoin	128	R
Cefepime	<= 1	S	Trimethoprim/Sulfamethoxazole	80	R
Ertapenem	<= 0.5	S			

+= Deduced drug *= AES modified **= User modified

AES Findings		
Confidence:	Consistent with correction	
Phenotypes flagged forreview:	QUINOLONES	PARTIALLY RESISTANT

Appendix 3

Cas1-1

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-89	59	CpG
3	BstUI - BstUI	90-150	61	CpG
4	BstUI - BstUI	151-211	61	CpG
5	BstUI - BstUI	212-389	178	CpG
6	BstUI - BstUI	390-395	6	CpG
7	BstUI - BstUI	396-519	124	CpG
8	BstUI - BstUI	520-580	61	CpG
9	BstUI - BstUI	581-641	61	CpG
10	BstUI - BstUI	642-702	61	CpG
11	BstUI - BstUI	703-763	61	CpG
12	BstUI - BstUI	764-805	42	CpG
13	BstUI - BstUI	806-824	19	CpG
14	BstUI - BstUI	825-887	63	CpG
15	BstUI - BstUI	888-929	42	CpG
16	BstUI - BstUI	930-948	19	CpG
17	BstUI - BstUI	949-1009	61	CpG
18	BstUI - BstUI	1010-1070	61	CpG
19	BstUI - BstUI	1071-1131	61	CpG
20	BstUI - BstUI	1132-1154	23	CpG
21	BstUI - BstUI	1155-1192	38	CpG
22	BstUI - (RightEnd)	1193-1216	24	CpG

Cas1-2

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-88	58	CpG
3	BstUI - BstUI	89-149	61	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
4	BstUI - BstUI	150-231	82	CpG
5	BstUI - BstUI	232-250	19	CpG
6	BstUI - BstUI	251-311	61	CpG
7	BstUI - BstUI	312-372	61	CpG
8	BstUI - BstUI	373-433	61	CpG
9	BstUI - BstUI	434-494	61	CpG
10	BstUI - BstUI	495-555	61	CpG
11	BstUI - BstUI	556-616	61	CpG
12	BstUI - BstUI	617-677	61	CpG
13	BstUI - BstUI	678-1137	460	CpG
14	BstUI - BstUI	1138-1266	129	CpG
15	BstUI - BstUI	1267-1317	51	CpG
16	BstUI - BstUI	1318-1323	6	CpG
17	BstUI - BstUI	1324-1342	19	CpG
18	BstUI - BstUI	1343-1357	15	CpG
19	BstUI - BstUI	1358-1403	46	CpG
20	BstUI - (RightEnd)	1404-1442	39	CpG

Cas1-3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-87	57	CpG
3	BstUI - BstUI	88-148	61	CpG
4	BstUI - BstUI	149-209	61	CpG
5	BstUI - BstUI	210-231	22	CpG
6	BstUI - BstUI	232-270	39	CpG
7	BstUI - BstUI	271-331	61	CpG
8	BstUI - BstUI	332-392	61	CpG
9	BstUI - BstUI	393-453	61	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
10	BstUI - BstUI	454-514	61	CpG
11	BstUI - BstUI	515-540	26	CpG
12	BstUI - BstUI	541-575	35	CpG
13	BstUI - BstUI	576-636	61	CpG
14	BstUI - BstUI	637-697	61	CpG
15	BstUI - BstUI	698-1178	481	CpG
16	BstUI - BstUI	1179-1268	90	CpG
17	BstUI - BstUI	1269-1340	72	CpG
18	BstUI - BstUI	1341-1355	15	CpG
19	BstUI - BstUI	1356-1401	46	CpG
20	BstUI - (RightEnd)	1402-1440	39	CpG

Cas1-4

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-90	60	CpG
3	BstUI - BstUI	91-151	61	CpG
4	BstUI - BstUI	152-212	61	CpG
5	BstUI - BstUI	213-273	61	CpG
6	BstUI - BstUI	274-334	61	CpG
7	BstUI - BstUI	335-395	61	CpG
8	BstUI - BstUI	396-420	25	CpG
9	BstUI - BstUI	421-456	36	CpG
10	BstUI - BstUI	457-517	61	CpG
11	BstUI - (RightEnd)	518-761	244	CpG

Cas1-5

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-91	91	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
2	BstUI - BstUI	92-152	61	CpG
3	BstUI - BstUI	153-213	61	CpG
4	BstUI - BstUI	214-274	61	CpG
5	BstUI - BstUI	275-335	61	CpG
6	BstUI - BstUI	336-396	61	CpG
7	BstUI - BstUI	397-421	25	CpG
8	BstUI - BstUI	422-457	36	CpG
9	BstUI - BstUI	458-518	61	CpG
10	BstUI - BstUI	519-717	199	CpG
11	BstUI - (RightEnd)	718-758	41	CpG

Cas1-6

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-87	87	CpG
2	BstUI - BstUI	88-148	61	CpG
3	BstUI - BstUI	149-209	61	CpG
4	BstUI - BstUI	210-231	22	CpG
5	BstUI - BstUI	232-270	39	CpG
6	BstUI - BstUI	271-331	61	CpG
7	BstUI - BstUI	332-392	61	CpG
8	BstUI - BstUI	393-453	61	CpG
9	BstUI - BstUI	454-514	61	CpG
10	BstUI - BstUI	515-540	26	CpG
11	BstUI - BstUI	541-575	35	CpG
12	BstUI - BstUI	576-636	61	CpG
13	BstUI - BstUI	637-822	186	CpG
14	BstUI - BstUI	823-841	19	CpG
15	BstUI - BstUI	842-904	63	CpG
16	BstUI - BstUI	905-946	42	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
17	BstUI - BstUI	947-965	19	CpG
18	BstUI - BstUI	966-1026	61	CpG
19	BstUI - BstUI	1027-1087	61	CpG
20	BstUI - BstUI	1088-1148	61	CpG
21	BstUI - BstUI	1149-1171	23	CpG
22	BstUI - BstUI	1172-1209	38	CpG
23	BstUI - (RightEnd)	1210-1233	24	CpG

Cas1-7

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-32	2	CpG
3	BstUI - BstUI	33-93	61	CpG
4	BstUI - BstUI	94-135	42	CpG
5	BstUI - BstUI	136-154	19	CpG
6	BstUI - BstUI	155-215	61	CpG
7	BstUI - BstUI	216-276	61	CpG
8	BstUI - BstUI	277-337	61	CpG
9	BstUI - BstUI	338-379	42	CpG
10	BstUI - BstUI	380-398	19	CpG
11	BstUI - BstUI	399-459	61	CpG
12	BstUI - BstUI	460-520	61	CpG
13	BstUI - BstUI	521-581	61	CpG
14	BstUI - BstUI	582-642	61	CpG
15	BstUI - BstUI	643-704	62	CpG
16	BstUI - BstUI	705-868	164	CpG
17	BstUI - BstUI	869-888	20	CpG
18	BstUI - BstUI	889-930	42	CpG
19	BstUI - BstUI	931-949	19	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
20	BstUI - BstUI	950-1014	65	CpG
21	BstUI - (RightEnd)	1015-1022	8	CpG

Cas1-8

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-92	92	CpG
2	BstUI - BstUI	93-134	42	CpG
3	BstUI - BstUI	135-153	19	CpG
4	BstUI - BstUI	154-214	61	CpG
5	BstUI - BstUI	215-275	61	CpG
6	BstUI - BstUI	276-336	61	CpG
7	BstUI - BstUI	337-378	42	CpG
8	BstUI - BstUI	379-397	19	CpG
9	BstUI - BstUI	398-458	61	CpG
10	BstUI - BstUI	459-519	61	CpG
11	BstUI - BstUI	520-580	61	CpG
12	BstUI - BstUI	581-641	61	CpG
13	BstUI - BstUI	642-703	62	CpG
14	BstUI - BstUI	704-886	183	CpG
15	BstUI - BstUI	887-928	42	CpG
16	BstUI - BstUI	929-947	19	CpG
17	BstUI - BstUI	948-1012	65	CpG
18	BstUI - (RightEnd)	1013-1020	8	CpG

Cas 1-9

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-44	44	CpG
2	BstUI - BstUI	45-84	40	CpG
3	BstUI - BstUI	85-92	8	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
4	BstUI - BstUI	93-133	41	CpG
5	BstUI - BstUI	134-152	19	CpG
6	BstUI - BstUI	153-213	61	CpG
7	BstUI - BstUI	214-274	61	CpG
8	BstUI - BstUI	275-335	61	CpG
9	BstUI - BstUI	336-377	42	CpG
10	BstUI - BstUI	378-396	19	CpG
11	BstUI - BstUI	397-457	61	CpG
12	BstUI - BstUI	458-518	61	CpG
13	BstUI - BstUI	519-579	61	CpG
14	BstUI - BstUI	580-640	61	CpG
15	BstUI - BstUI	641-883	243	CpG
16	BstUI - (RightEnd)	884-1009	126	CpG

Cas1-10

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-90	90	CpG
2	BstUI - BstUI	91-151	61	CpG
3	BstUI - BstUI	152-212	61	CpG
4	BstUI - BstUI	213-273	61	CpG
5	BstUI - BstUI	274-334	61	CpG
6	BstUI - BstUI	335-395	61	CpG
7	BstUI - BstUI	396-456	61	CpG
8	BstUI - BstUI	457-517	61	CpG
9	BstUI - BstUI	518-578	61	CpG
10	BstUI - BstUI	579-639	61	CpG
11	BstUI - BstUI	640-701	62	CpG
12	BstUI - BstUI	702-762	61	CpG
13	BstUI - BstUI	763-797	35	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
14	BstUI - BstUI	798-823	26	CpG
15	BstUI - (RightEnd)	824-1004	181	CpG

Cas1-11

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-90	90	CpG
2	BstUI - BstUI	91-132	42	CpG
3	BstUI - BstUI	133-151	19	CpG
4	BstUI - BstUI	152-212	61	CpG
5	BstUI - BstUI	213-273	61	CpG
6	BstUI - BstUI	274-334	61	CpG
7	BstUI - BstUI	335-376	42	CpG
8	BstUI - BstUI	377-395	19	CpG
9	BstUI - BstUI	396-456	61	CpG
10	BstUI - BstUI	457-517	61	CpG
11	BstUI - BstUI	518-578	61	CpG
12	BstUI - BstUI	579-639	61	CpG
13	BstUI - BstUI	640-701	62	CpG
14	BstUI - BstUI	702-892	191	CpG
15	BstUI - (RightEnd)	893-1005	113	CpG

Cas1-12

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-91	91	CpG
2	BstUI - BstUI	92-133	42	CpG
3	BstUI - BstUI	134-335	202	CpG
4	BstUI - BstUI	336-377	42	CpG
5	BstUI - BstUI	378-396	19	CpG
6	BstUI - BstUI	397-457	61	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
7	BstUI - BstUI	458-518	61	CpG
8	BstUI - BstUI	519-638	120	CpG
9	BstUI - BstUI	639-700	62	CpG
10	BstUI - BstUI	701-891	191	CpG
11	BstUI - (RightEnd)	892-1004	113	CpG

Cas1-13

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-140	110	CpG
3	BstUI - BstUI	141-153	13	CpG
4	BstUI - BstUI	154-173	20	CpG
5	BstUI - BstUI	174-201	28	CpG
6	BstUI - BstUI	202-262	61	CpG
7	BstUI - BstUI	263-302	40	CpG
8	BstUI - BstUI	303-304	2	CpG
9	BstUI - BstUI	305-323	19	CpG
10	BstUI - BstUI	324-419	96	CpG
11	BstUI - BstUI	420-445	26	CpG
12	BstUI - BstUI	446-471	26	CpG
13	BstUI - (RightEnd)	472-763	292	CpG

Cas1-14

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-141	111	CpG
3	BstUI - BstUI	142-154	13	CpG
4	BstUI - BstUI	155-174	20	CpG
5	BstUI - BstUI	175-202	28	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
6	BstUI - BstUI	203-263	61	CpG
7	BstUI - BstUI	264-303	40	CpG
8	BstUI - BstUI	304-305	2	CpG
9	BstUI - BstUI	306-324	19	CpG
10	BstUI - BstUI	325-420	96	CpG
11	BstUI - BstUI	421-446	26	CpG
12	BstUI - BstUI	447-472	26	CpG
13	BstUI - BstUI	473-645	173	CpG
14	BstUI - (RightEnd)	646-778	133	CpG

Cas1-15

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-142	112	CpG
3	BstUI - BstUI	143-155	13	CpG
4	BstUI - BstUI	156-175	20	CpG
5	BstUI - BstUI	176-203	28	CpG
6	BstUI - BstUI	204-264	61	CpG
7	BstUI - BstUI	265-304	40	CpG
8	BstUI - BstUI	305-306	2	CpG
9	BstUI - BstUI	307-325	19	CpG
10	BstUI - BstUI	326-421	96	CpG
11	BstUI - BstUI	422-447	26	CpG
12	BstUI - BstUI	448-473	26	CpG
13	BstUI - (RightEnd)	474-794	321	CpG

Cas1-16

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-143	113	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
3	BstUI - BstUI	144-156	13	CpG
4	BstUI - BstUI	157-176	20	CpG
5	BstUI - BstUI	177-204	28	CpG
6	BstUI - BstUI	205-265	61	CpG
7	BstUI - BstUI	266-305	40	CpG
8	BstUI - BstUI	306-307	2	CpG
9	BstUI - BstUI	308-326	19	CpG
10	BstUI - BstUI	327-422	96	CpG
11	BstUI - BstUI	423-448	26	CpG
12	BstUI - BstUI	449-474	26	CpG
13	BstUI - BstUI	475-706	232	CpG
14	BstUI - BstUI	707-792	86	CpG
15	BstUI - (RightEnd)	793-808	16	CpG

Cas1-17

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-138	108	CpG
3	BstUI - BstUI	139-151	13	CpG
4	BstUI - BstUI	152-171	20	CpG
5	BstUI - BstUI	172-199	28	CpG
6	BstUI - BstUI	200-260	61	CpG
7	BstUI - BstUI	261-300	40	CpG
8	BstUI - BstUI	301-302	2	CpG
9	BstUI - BstUI	303-321	19	CpG
10	BstUI - BstUI	322-417	96	CpG
11	BstUI - BstUI	418-443	26	CpG
12	BstUI - BstUI	444-469	26	CpG
13	BstUI - (RightEnd)	470-799	330	CpG

Appendix 3

Cas1-18

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-30	30	CpG
2	BstUI - BstUI	31-140	110	CpG
3	BstUI - BstUI	141-153	13	CpG
4	BstUI - BstUI	154-173	20	CpG
5	BstUI - BstUI	174-201	28	CpG
6	BstUI - BstUI	202-262	61	CpG
7	BstUI - BstUI	263-302	40	CpG
8	BstUI - BstUI	303-304	2	CpG
9	BstUI - BstUI	305-323	19	CpG
10	BstUI - BstUI	324-419	96	CpG
11	BstUI - BstUI	420-445	26	CpG
12	BstUI - BstUI	446-471	26	CpG
13	BstUI - BstUI	472-644	173	CpG
14	BstUI - BstUI	645-676	32	CpG
15	BstUI - BstUI	677-687	11	CpG
16	BstUI - BstUI	688-696	9	CpG
17	BstUI - BstUI	697-752	56	CpG
18	BstUI - (RightEnd)	753-796	44	CpG

Cas2-1

Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-44	44
2	BstUI - BstUI	45-124	80
3	BstUI - BstUI	125-185	61
4	BstUI - BstUI	186-246	61
5	BstUI - BstUI	247-307	61
6	BstUI - BstUI	308-368	61

Appendix 3

Ends	Coordinates	Length (bp)	Affected by Methylation
7	BstUI - BstUI	369-429	61
8	BstUI - BstUI	430-615	186
9	BstUI - BstUI	616-651	36
10	BstUI - BstUI	652-656	5
11	BstUI - BstUI	657-675	19
12	BstUI - BstUI	676-689	14
13	BstUI - (RightEnd)	690-692	3

Cas2-2

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-44	44	CpG
2	BstUI - BstUI	45-124	80	CpG
3	BstUI - BstUI	125-185	61	CpG
4	BstUI - BstUI	186-196	11	CpG
5	BstUI - BstUI	197-206	10	CpG
6	BstUI - BstUI	207-246	40	CpG
7	BstUI - BstUI	247-288	42	CpG
8	BstUI - BstUI	289-307	19	CpG
9	BstUI - BstUI	308-368	61	CpG
10	BstUI - BstUI	369-429	61	CpG
11	BstUI - BstUI	430-490	61	CpG
12	BstUI - BstUI	491-551	61	CpG
13	BstUI - BstUI	552-612	61	CpG
14	BstUI - BstUI	613-655	43	CpG
15	BstUI - BstUI	656-674	19	CpG
16	BstUI - BstUI	675-735	61	CpG
17	BstUI - BstUI	736-796	61	CpG
18	BstUI - BstUI	797-857	61	CpG
19	BstUI - BstUI	858-918	61	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
20	BstUI - BstUI	919-979	61	CpG
21	BstUI - BstUI	980-1008	29	CpG
22	BstUI - BstUI	1009-1040	32	CpG
23	BstUI - BstUI	1041-1102	62	CpG
24	BstUI - BstUI	1103-1163	61	CpG
25	BstUI - BstUI	1164-1261	98	CpG
26	BstUI - BstUI	1262-1348	87	CpG
27	BstUI - BstUI	1349-1409	61	CpG
28	BstUI - BstUI	1410-1470	61	CpG
29	BstUI - BstUI	1471-1531	61	CpG
30	BstUI - BstUI	1532-1592	61	CpG
31	BstUI - BstUI	1593-1606	14	CpG
32	BstUI - BstUI	1607-1653	47	CpG
33	BstUI - BstUI	1654-1664	11	CpG
34	BstUI - BstUI	1665-1714	50	CpG
35	BstUI - BstUI	1715-1731	17	CpG
36	BstUI - BstUI	1732-1775	44	CpG
37	BstUI - BstUI	1776-1836	61	CpG
38	BstUI - BstUI	1837-1897	61	CpG
39	BstUI - BstUI	1898-1958	61	CpG
40	BstUI - (RightEnd)	1959-1982	24	CpG

Cas2-3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-42	42	CpG
2	BstUI - BstUI	43-122	80	CpG
3	BstUI - BstUI	123-183	61	CpG
4	BstUI - BstUI	184-244	61	CpG
5	BstUI - BstUI	245-255	11	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
6	BstUI - BstUI	256-305	50	CpG
7	BstUI - BstUI	306-366	61	CpG
8	BstUI - BstUI	367-408	42	CpG
9	BstUI - BstUI	409-466	58	CpG
10	BstUI - BstUI	467-488	22	CpG
11	BstUI - BstUI	489-519	31	CpG
12	BstUI - BstUI	520-530	11	CpG
13	BstUI - BstUI	531-549	19	CpG
14	BstUI - BstUI	550-610	61	CpG
15	BstUI - BstUI	611-671	61	CpG
16	BstUI - BstUI	672-732	61	CpG
17	BstUI - BstUI	733-793	61	CpG
18	BstUI - BstUI	794-854	61	CpG
19	BstUI - BstUI	855-915	61	CpG
20	BstUI - BstUI	916-957	42	CpG
21	BstUI - BstUI	958-976	19	CpG
22	BstUI - BstUI	977-1004	28	CpG
23	BstUI - BstUI	1005-1037	33	CpG
24	BstUI - BstUI	1038-1156	119	CpG
25	BstUI - BstUI	1157-1229	73	CpG
26	BstUI - BstUI	1230-1288	59	CpG
27	BstUI - BstUI	1289-1349	61	CpG
28	BstUI - BstUI	1350-1410	61	CpG
29	BstUI - BstUI	1411-1471	61	CpG
30	BstUI - BstUI	1472-1532	61	CpG
31	BstUI - BstUI	1533-1593	61	CpG
32	BstUI - BstUI	1594-1607	14	CpG
33	BstUI - BstUI	1608-1654	47	CpG
34	BstUI - BstUI	1655-1665	11	CpG
35	BstUI - BstUI	1666-1715	50	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
36	BstUI - BstUI	1716-1732	17	CpG
37	BstUI - BstUI	1733-1776	44	CpG
38	BstUI - BstUI	1777-1837	61	CpG
39	BstUI - BstUI	1838-1898	61	CpG
40	BstUI - BstUI	1899-1959	61	CpG
41	BstUI - (RightEnd)	1960-1980	21	CpG

Cas2-4

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-38	38	CpG
2	BstUI - BstUI	39-118	80	CpG
3	BstUI - BstUI	119-179	61	CpG
4	BstUI - BstUI	180-301	122	CpG
5	BstUI - BstUI	302-362	61	CpG
6	BstUI - BstUI	363-423	61	CpG
7	BstUI - BstUI	424-484	61	CpG
8	BstUI - BstUI	485-545	61	CpG
9	BstUI - BstUI	546-559	14	CpG
10	BstUI - BstUI	560-606	47	CpG
11	BstUI - BstUI	607-667	61	CpG
12	BstUI - BstUI	668-728	61	CpG
13	BstUI - BstUI	729-789	61	CpG
14	BstUI - BstUI	790-831	42	CpG
15	BstUI - BstUI	832-850	19	CpG
16	BstUI - BstUI	851-892	42	CpG
17	BstUI - BstUI	893-911	19	CpG
18	BstUI - BstUI	912-972	61	CpG
19	BstUI - BstUI	973-999	27	CpG
20	BstUI - BstUI	1000-1014	15	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
21	BstUI - BstUI	1015-1033	19	CpG
22	BstUI - BstUI	1034-1358	325	CpG
23	BstUI - BstUI	1359-1419	61	CpG
24	BstUI - (RightEnd)	1420-1433	14	CpG

Cas2-5

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-44	44	CpG
2	BstUI - BstUI	45-124	80	CpG
3	BstUI - BstUI	125-185	61	CpG
4	BstUI - BstUI	186-288	103	CpG
5	BstUI - BstUI	289-307	19	CpG
6	BstUI - BstUI	308-368	61	CpG
7	BstUI - BstUI	369-387	19	CpG
8	BstUI - BstUI	388-389	2	CpG
9	BstUI - BstUI	390-430	41	CpG
10	BstUI - BstUI	431-491	61	CpG
11	BstUI - BstUI	492-552	61	CpG
12	BstUI - BstUI	553-613	61	CpG
13	BstUI - BstUI	614-674	61	CpG
14	BstUI - BstUI	675-704	30	CpG
15	BstUI - BstUI	705-735	31	CpG
16	BstUI - BstUI	736-943	208	CpG
17	BstUI - BstUI	944-1012	69	CpG
18	BstUI - (RightEnd)	1013-1051	39	CpG

Cas2-6

#	Ends	Coordinates	Length (bp)	Affected by Methylation

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-45	45	CpG
2	BstUI - BstUI	46-124	79	CpG
3	BstUI - BstUI	125-185	61	CpG
4	BstUI - BstUI	186-246	61	CpG
5	BstUI - BstUI	247-257	11	CpG
6	BstUI - BstUI	258-307	50	CpG
7	BstUI - BstUI	308-368	61	CpG
8	BstUI - BstUI	369-410	42	CpG
9	BstUI - BstUI	411-468	58	CpG
10	BstUI - BstUI	469-490	22	CpG
11	BstUI - BstUI	491-521	31	CpG
12	BstUI - BstUI	522-532	11	CpG
13	BstUI - BstUI	533-551	19	CpG
14	BstUI - BstUI	552-612	61	CpG
15	BstUI - BstUI	613-673	61	CpG
16	BstUI - BstUI	674-734	61	CpG
17	BstUI - BstUI	735-795	61	CpG
18	BstUI - BstUI	796-856	61	CpG
19	BstUI - BstUI	857-917	61	CpG
20	BstUI - BstUI	918-959	42	CpG
21	BstUI - BstUI	960-978	19	CpG
22	BstUI - BstUI	979-1006	28	CpG
23	BstUI - BstUI	1007-1039	33	CpG
24	BstUI - BstUI	1040-1100	61	CpG
25	BstUI - BstUI	1101-1302	202	CpG
26	BstUI - BstUI	1303-1363	61	CpG
27	BstUI - BstUI	1364-1424	61	CpG
28	BstUI - BstUI	1425-1485	61	CpG
29	BstUI - BstUI	1486-1546	61	CpG
30	BstUI - BstUI	1547-1607	61	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
31	BstUI - BstUI	1608-1621	14	CpG
32	BstUI - BstUI	1622-1668	47	CpG
33	BstUI - BstUI	1669-1679	11	CpG
34	BstUI - BstUI	1680-1729	50	CpG
35	BstUI - BstUI	1730-1746	17	CpG
36	BstUI - BstUI	1747-1790	44	CpG
37	BstUI - BstUI	1791-1851	61	CpG
38	BstUI - BstUI	1852-1912	61	CpG
39	BstUI - (RightEnd)	1913-1935	23	CpG

Cas2-7

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-44	44	CpG
2	BstUI - BstUI	45-124	80	CpG
3	BstUI - BstUI	125-150	26	CpG
4	BstUI - BstUI	151-185	35	CpG
5	BstUI - BstUI	186-246	61	CpG
6	BstUI - BstUI	247-307	61	CpG
7	BstUI - BstUI	308-368	61	CpG
8	BstUI - BstUI	369-410	42	CpG
9	BstUI - BstUI	411-429	19	CpG
10	BstUI - BstUI	430-490	61	CpG
11	BstUI - BstUI	491-551	61	CpG
12	BstUI - BstUI	552-612	61	CpG
13	BstUI - BstUI	613-654	42	CpG
14	BstUI - BstUI	655-673	19	CpG
15	BstUI - BstUI	674-734	61	CpG
16	BstUI - BstUI	735-776	42	CpG
17	BstUI - BstUI	777-795	19	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
18	BstUI - BstUI	796-856	61	CpG
19	BstUI - BstUI	857-917	61	CpG
20	BstUI - BstUI	918-944	27	CpG
21	BstUI - BstUI	945-978	34	CpG
22	BstUI - BstUI	979-1039	61	CpG
23	BstUI - (RightEnd)	1040-1094	55	CpG

Cas2-8

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-43	43	CpG
2	BstUI - BstUI	44-123	80	CpG
3	BstUI - BstUI	124-149	26	CpG
4	BstUI - BstUI	150-184	35	CpG
5	BstUI - BstUI	185-245	61	CpG
6	BstUI - BstUI	246-306	61	CpG
7	BstUI - BstUI	307-367	61	CpG
8	BstUI - BstUI	368-409	42	CpG
9	BstUI - BstUI	410-428	19	CpG
10	BstUI - BstUI	429-489	61	CpG
11	BstUI - BstUI	490-550	61	CpG
12	BstUI - BstUI	551-611	61	CpG
13	BstUI - BstUI	612-653	42	CpG
14	BstUI - BstUI	654-672	19	CpG
15	BstUI - BstUI	673-733	61	CpG
16	BstUI - BstUI	734-775	42	CpG
17	BstUI - BstUI	776-794	19	CpG
18	BstUI - BstUI	795-855	61	CpG
19	BstUI - BstUI	856-916	61	CpG
20	BstUI - BstUI	917-943	27	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
21	BstUI - BstUI	944-977	34	CpG
22	BstUI - BstUI	978-1038	61	CpG
23	BstUI - (RightEnd)	1039-1100	62	CpG

Cas2-9

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-44	44	CpG
2	BstUI - BstUI	45-123	79	CpG
3	BstUI - BstUI	124-149	26	CpG
4	BstUI - BstUI	150-184	35	CpG
5	BstUI - BstUI	185-245	61	CpG
6	BstUI - BstUI	246-306	61	CpG
7	BstUI - BstUI	307-367	61	CpG
8	BstUI - BstUI	368-409	42	CpG
9	BstUI - BstUI	410-428	19	CpG
10	BstUI - BstUI	429-489	61	CpG
11	BstUI - BstUI	490-550	61	CpG
12	BstUI - BstUI	551-611	61	CpG
13	BstUI - BstUI	612-653	42	CpG
14	BstUI - BstUI	654-672	19	CpG
15	BstUI - BstUI	673-733	61	CpG
16	BstUI - BstUI	734-775	42	CpG
17	BstUI - BstUI	776-794	19	CpG
18	BstUI - BstUI	795-855	61	CpG
19	BstUI - BstUI	856-916	61	CpG
20	BstUI - BstUI	917-943	27	CpG
21	BstUI - BstUI	944-977	34	CpG
22	BstUI - BstUI	978-1038	61	CpG
23	BstUI - BstUI	1039-1144	106	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
24	BstUI - (RightEnd)	1145-1298	154	CpG

Cas2-10

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-42	42	CpG
2	BstUI - BstUI	43-122	80	CpG
3	BstUI - BstUI	123-183	61	CpG
4	BstUI - BstUI	184-244	61	CpG
5	BstUI - BstUI	245-286	42	CpG
6	BstUI - BstUI	287-305	19	CpG
7	BstUI - BstUI	306-366	61	CpG
8	BstUI - BstUI	367-427	61	CpG
9	BstUI - BstUI	428-488	61	CpG
10	BstUI - BstUI	489-549	61	CpG
11	BstUI - BstUI	550-610	61	CpG
12	BstUI - BstUI	611-671	61	CpG
13	BstUI - BstUI	672-732	61	CpG
14	BstUI - BstUI	733-793	61	CpG
15	BstUI - BstUI	794-854	61	CpG
16	BstUI - BstUI	855-896	42	CpG
17	BstUI - BstUI	897-915	19	CpG
18	BstUI - BstUI	916-977	62	CpG
19	BstUI - BstUI	978-1038	61	CpG
20	BstUI - BstUI	1039-1101	63	CpG
21	BstUI - BstUI	1102-1103	2	CpG
22	BstUI - BstUI	1104-1161	58	CpG
23	BstUI - BstUI	1162-1304	143	CpG
24	BstUI - BstUI	1305-1365	61	CpG
25	BstUI - BstUI	1366-1426	61	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
26	BstUI - BstUI	1427-1487	61	CpG
27	BstUI - BstUI	1488-1548	61	CpG
28	BstUI - BstUI	1549-1609	61	CpG
29	BstUI - BstUI	1610-1623	14	CpG
30	BstUI - BstUI	1624-1670	47	CpG
31	BstUI - BstUI	1671-1681	11	CpG
32	BstUI - BstUI	1682-1731	50	CpG
33	BstUI - BstUI	1732-1748	17	CpG
34	BstUI - BstUI	1749-1792	44	CpG
35	BstUI - BstUI	1793-1853	61	CpG
36	BstUI - BstUI	1854-1914	61	CpG
37	BstUI - BstUI	1915-1975	61	CpG
38	BstUI - (RightEnd)	1976-2020	45	CpG

Cas2-11

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-42	42	CpG
2	BstUI - BstUI	43-122	80	CpG
3	BstUI - BstUI	123-148	26	CpG
4	BstUI - BstUI	149-183	35	CpG
5	BstUI - BstUI	184-244	61	CpG
6	BstUI - BstUI	245-305	61	CpG
7	BstUI - BstUI	306-366	61	CpG
8	BstUI - BstUI	367-408	42	CpG
9	BstUI - BstUI	409-427	19	CpG
10	BstUI - BstUI	428-488	61	CpG
11	BstUI - BstUI	489-549	61	CpG
12	BstUI - BstUI	550-610	61	CpG
13	BstUI - BstUI	611-652	42	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
14	BstUI - BstUI	653-671	19	CpG
15	BstUI - BstUI	672-732	61	CpG
16	BstUI - BstUI	733-774	42	CpG
17	BstUI - BstUI	775-793	19	CpG
18	BstUI - BstUI	794-854	61	CpG
19	BstUI - BstUI	855-915	61	CpG
20	BstUI - BstUI	916-942	27	CpG
21	BstUI - BstUI	943-976	34	CpG
22	BstUI - BstUI	977-1037	61	CpG
23	BstUI - BstUI	1038-1098	61	CpG
24	BstUI - BstUI	1099-1140	42	CpG
25	BstUI - BstUI	1141-1284	144	CpG
26	BstUI - BstUI	1285-1345	61	CpG
27	BstUI - BstUI	1346-1406	61	CpG
28	BstUI - (RightEnd)	1407-1456	50	CpG

Cas2-12

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-42	42	CpG
2	BstUI - BstUI	43-122	80	CpG
3	BstUI - BstUI	123-148	26	CpG
4	BstUI - BstUI	149-183	35	CpG
5	BstUI - BstUI	184-244	61	CpG
6	BstUI - BstUI	245-305	61	CpG
7	BstUI - BstUI	306-366	61	CpG
8	BstUI - BstUI	367-408	42	CpG
9	BstUI - BstUI	409-427	19	CpG
10	BstUI - BstUI	428-488	61	CpG
11	BstUI - BstUI	489-549	61	CpG

Appendix 3

#	Ends	Coordinates	Length (bp)	Affected by Methylation
12	BstUI - BstUI	550-610	61	CpG
13	BstUI - BstUI	611-652	42	CpG
14	BstUI - BstUI	653-671	19	CpG
15	BstUI - BstUI	672-732	61	CpG
16	BstUI - BstUI	733-774	42	CpG
17	BstUI - BstUI	775-793	19	CpG
18	BstUI - BstUI	794-854	61	CpG
19	BstUI - BstUI	855-915	61	CpG
20	BstUI - BstUI	916-942	27	CpG
21	BstUI - BstUI	943-976	34	CpG
22	BstUI - BstUI	977-1037	61	CpG
23	BstUI - BstUI	1038-1098	61	CpG
24	BstUI - BstUI	1099-1138	40	CpG
25	BstUI - BstUI	1139-1281	143	CpG
26	BstUI - BstUI	1282-1342	61	CpG
27	BstUI - BstUI	1343-1403	61	CpG
28	BstUI - (RightEnd)	1404-1453	50	CpG

Cas2-13

Ends	Coordinates	Length (bp)	Affected by Methylation
1	(LeftEnd) - BstUI	1-42	42
2	BstUI - BstUI	43-122	80
3	BstUI - BstUI	123-183	61
4	BstUI - BstUI	184-286	103
5	BstUI - BstUI	287-305	19
6	BstUI - BstUI	306-366	61
7	BstUI - BstUI	367-385	19
8	BstUI - BstUI	386-387	2
9	BstUI - BstUI	388-428	41

Appendix 3

Ends	Coordinates	Length (bp)	Affected by Methylation
10	BstUI - BstUI	429-489	61
11	BstUI - BstUI	490-550	61
12	BstUI - BstUI	551-611	61
13	BstUI - BstUI	612-672	61
14	BstUI - BstUI	673-702	30
15	BstUI - BstUI	703-733	31
16	BstUI - BstUI	734-872	139
17	BstUI - (RightEnd)	873-1248	376

الخلاصة:

خلال فترة الدراسة تم جمع 200 عينة سريرية براز و دم ، عينات البراز تم اخذها من المرضى الذين يعانون من الاسهال المائي الذي يصاحبه احيانا مادة مخاطية او قيح او قليل من الدم وعينات الدم تم اخذها من مرضى التايفوئيد لكلا الجنسين ولكافة الاعمار. وكانوا المرضى من المراجعين الى المستشفيات الرئيسة في محافظة بابل هي: مستشفى الامام الصادق (ع) و مستشفى الحلة الجراحي التعليمي ومستشفى بابل التعليمي للنسائية والاطفال، خلال فترة اربعة اشهر (من شباط 2022 الى ايار 2022). بعد ذلك جرى زرع هذه العينات في وسط انتقائي تم تحديده من خلال استخدام فحوصات حيوية كيميائية وبكتريولوجية (نظام فيتك 2 المدمج)، وباستخدام بادئ خاص (SE1472298-2 للسالمونيلا انترايتدس ، STM4497 للسالمونيلا تايفيموريوم و *tyv* O antigen synthesis للسالمونيلا تايفي).

من بين 200 عينة ، تم الكشف عن 34 (17٪) عزلة فقط من *S. enterica* عن طريق الزرع و نظام الفيتك 2 المضغوط. وبعد ذلك باستخدام جين خاص ، أظهرت النتائج أن 25 عزلة (73.53٪) فقط أعطت نتيجة موجبة لهذه الجينات تضمنت 13 (52)٪ من *S. enteritidis* ، و 6 (24)٪ من *S. typhimurium* و 6 (24)٪ من العزلات. *S. typhi*.

وتم القيام باختبار التحسس للمضادات الحياتية عن طريق الفيتك 2 المدمج (نوع البطاقة AST GN76) للكشف عن الحساسية المكروبية ومقاومة عزلات السالمونيلا المعوية. وقد كانت المضادات الحياتية المستخدمة في هذه الدراسة حساسة للعزلات. حيث كانت العزلات حساسة بدرجة عالية للمضادات الحيوية ترايميثوبرين/ سالفاميثوكزازول، و امبنيم و تيكيسايكلن وبنسبة

100%، تتبعها المضادات بيبراسلين/تازوباكتام و سفيبيم و ارتابينيم بنسبة 96%،92%،92% على التوالي وايضا سفتازيديم و سفترياكزون بنسبة 82%. الا ان العزلات اظهرت معدل حساسية معتدلة (64%، 64%، 52%) تجاه الامبسلين والسبروفلوكساسين و النترفيورينشن، على التوالي. وكذلك اظهرت النتائج بان بعض العزلات كانت مقاومة بنسبة 88% للسيفازولين والسيفوكستين و الليفوفلوكساسين. كما ان العزلات كانت مقاوِمة للاميكاسين و الجنتيمايسين بنسبة مقاومة تصل الى 92%. وهذا يفسر بان هذه العقاقير لها نشاط جيد ضد السالمونيلا انتريكا ، لان اغلبها حساسة لهذه الادوية .

وشملت الدراسة الكشف عن التنوع الجيني بين العزلات البكتيرية الذي تم من خلال استخدام CRISPR)–PCR) وتم تقييمها لاحتمال وجودها في 18 من عزلات السالمونيلا المعويه .

تم إخضاع 18 من عزلات الأنماط المصلية للسالمونيلا المعوية المعزوله من عينات سريرية مختلفة (البراز و الدم) للكشف عن كريسبر 1 و 2 وقد أظهرت النتائج ان العزلات ال 18 (100٪) و 13 (72٪) كان PCR – موجب لكرسبر 1 و2 على التوالي. التكرار المباشر (DR) لعزلات كرسبر1 و كرسبر2 اظهرت طولا متطابقا 29 زوج قاعدي. أشار تعدد الأشكال ل DR لكرسبر 1 من خلال بناء شجرة النشوء والتطور إلى أن جميع الأنماط ال 18 يمكن تقسيمها إلى ستة خطوط (A–F) ، بينما من بين 13 نمطا متنوعا من كرسبر 2 يمكن تصنيفها إلى أربعة خطوط (A–D). كما تم إجراء قيمة التشابه الجيني للنمط المصلي للسالمونيلا المعوية وفقا لتسلسل التكرار لكرسبر 1 و كرسبر 2، وأظهرت النتائج أنه في كرسبر 1 هناك أقصى تشابه بين النمط المصلي بين SE11 و STM3 بنسبة (100٪) والحد

الأدنى من التشابه بين STM4 و STY29 بنسبة (40٪) ، بينما في كرسبر2 ، أقصى تشابه بين النمط المصلي SE11 وSTY29 بنسبة (100٪) و الحد الأدنى من التشابه بين النمط المصلي SE7 وSTM4 بنسبة (19٪).

من ناحية أخرى ، اختلف اختبار كريسبر الموجود في *السالمونيلا المعوية* في الطول وتمت دراسة spacer أيضا وأظهرت النتيجة أن عدد spacer لكرسبر 1 كان بين 13-5 spacer وكرسبر 2 كان بين 12-1 spacer. ومع ذلك ، تم إجراء مقارنة محاذاة تسلسل لكرسبر 1 وكرسبر 2 spacer في *السالمونيلا المعوية*. تظهر شجرة النشوء والتطور التي تم إنشاؤها من محاذاة تسلسل كرسبر 1 spacer أن كل نمط مصلي تم تقسيمه إلى مجموعة. على عكس كرسبر 1 ، لم تتمكن شجرة النشوء والتطور التي تم إنشاؤها من محاذاة تسلسل كرسبر 2 spacer من التمييز بين الانماط المصلية للسالمونيلا المعوية . كما تمت دراسة قيمة التشابه الوراثي للسالمونيلا المعوية (السالمونلا تايفي والسالمونيلا تايفيموريم) وفقا لتسلسل spacer ل كرسبر 1 و كرسبر 2 ، وأظهرت النتيجة أنه في كرسبر 1 هناك أقصى تشابه بين STM26 و STM2 بنسبة (100٪) والحد الأدنى من التشابه بين SE6 و STY29 بنسبة (11%) ، بينما في كرسبر 2 أقصى تشابه بين الأنماط المصلية STM4 و SE7 ، STM4 و SE8 ، STY29 وSE11 بنسبة (100٪) والحد الأدنى من التشابه بين الأنماط المصلية STM26 و SE6 بنسبة (10٪).

أظهرت دراستنا أيضا أنه في السيليكو PCR-RFLP من كريسبر ، أظهرت النتيجة أن تسلسل النوكليوتيدات ل كرسبر 1 و كرسبر 2 من الانماط المصلية للسالمونيلا المعوية الكشف

عن تنوع كبير في عدد ونوكليوتيدات spacer و DR. تراوحت القطع من 2–330 زوج قاعدي في كرسبر 1 ، بينما تراوحت القطع من 3–375 زوج قاعدي في كرسبر 2.

على المستوى الجينومي ، حاولت هذه الدراسة التمييز بين السلالات ذات الصلة ، السالمونيلا المعوية subsp. enterica serovar Typhimurium (STY9) والسالمونيلا المعوية subsp. enterica serovar Typhi (SalT33) ، باستخدام تسلسل الجينوم الكامل. لاحظت نتائج هذه الدراسة أن كروموسومات (STY9) و (SalT33) هي اختلافات طفيفة جدا ، حيث كان الحجم الجيني لSTY9 (4687295 bp ، 52.2٪ GC) أكبر من جينوم SalT33 (4679911 bp ، 52.12% GC). بعد تحليل الجينوم المقارن ، أظهرت جميع الجينومات المدروسة ل (STY9 و SalT33) أنماطا مختلفة من الأحداث التطورية (إعادة ترتيب الجينوم والكسب أو الخسارة المجزأة) مع بعضها البعض أو مع الجينوم المرجعي .

بالإضافة إلى ذلك ، تمت دراسة تشابه تسلسل الانماط المصلية للسالمونيلا المعوية بالمقارنة مع الجينوم المرجعي ، وكشفت نتيجة هذه الدراسة أن تشابه التسلسل في سلالة SalT33 كان أعلى من سلالة STY9 ، حيث يحتوي STY9 على فجوات أكثر من SalT33 بعد شرح هذه الجينومات ، أبلغت هذه الدراسة عن وجود (CDS 4701، tRNA 38 و 3 rRNA و 521 بروتين افتراضي) في جينوم STY9 بينما(CDS 4855 ، tRNA 68، 4 rRNA و 530 بروتين افتراضي) في جينوم SalT33.

تم شرح جميع بروتينات النظام الفرعي والبروتينات المتخصصة (الجينات المقاومة للمضادات الحيوية ، أهداف الدواء ، الناقلات وعوامل الفوعة) في هذه الدراسة ، حيث كان

هناك اختلاف طفيف بين STY9 و SalT33. وبالتالي ، تم تحديد البديل الذي يستدعي الانماط المصلية للسالمونيلا المعوية (STY9 و SalT33) بالمقارنة مع الجينوم المرجعي. وفقا للمتغيرات ، أظهر عدد الجينومات المدروسة وجود (25608) متغيرا شمل 99.01٪ (25355) SNPs و 0.53٪ (134) إدخال و 0.46٪ (119) حذف في جينوم STY9 و (680)متغيرا شمل 93.97٪ (639) SNPs، 2.95٪(20) إدخال و 3.08٪ (21) حذف في جينوم SalT33.

في المقابل ، تم حساب عدد التغيير الأساسي على كل SNPs لتحديد نوع اختلاف التسلسل. أظهرت جميع الجينومات المدروسة أنماط تباين التشابه ، حيث كانت الأنماط الأكثر شيوعا لاستبدال القاعدة هي بدائل T ↔ C و A ↔ G. تمثل هذه الأنماط استبدال الانتقال بدلا من استبدال التحويل في جميع الجينومات المدروسة بعد SNPs. في الختام ، توفر نتائج هذه الدراسة إطارا شاملا لعمل الجينوم الكامل للانماط المصلية للسالمونيلا المعوية.

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وزارة التعليم العالي والبحث العلمي

جامعة بابل كلية الطب

التغاير في الشجرة الوراثية ل CRISPR و تسلسل الجينوم الكلي للسالمونيلا *Salmonella enterica* المعزولة من عينات سريرية في محافظة بابل

اطروحة
مقدمة الى مجلس كليه الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في
العلوم/الأحياء المجهرية الطبية

من قبل

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ماجستير في الاحياء المجهرية الطبية كلية الطب / جامعة بابل 2019

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